

REMARKS/ARGUMENTS

Status of the Claims.

Claims 1, 3-6, 12-15, 17, 19-23, and 25 are currently pending in this application. Claims 1, 12, 14, and 17 are amended herein. The changes introduce no new matter and are fully supported by the application as filed. The current Office Action rejected claims 1, 3-6, 12, 13, 17, 19-23, and 25 as allegedly obvious under 35 U.S.C. §103(a) as to Kramer *et al.* (1995) in view of Urschel *et al.* (1990), Althaus (WO 9303140), Unger *et al.* (EP 731,108) and Unger *et al.* (1995) and in further view of Weiner *et al.* (USPN 5,935,577). Additionally, claims 14 and 15 were rejected under 35 U.S.C. §103(s) as allegedly obvious as to Kramer *et al.* (1995) in view of Urschel *et al.* (1990), Althaus (WO 9303140), Unger *et al.* (EP 731,108) and Unger *et al.* (1995) and in further view of Hammang *et al.* (USPN 5,904,144) and The Merck Manual (p. 1091). Applicants respectfully traverse.

Amendments to the claims.

In order to more clearly claim the current embodiment, claims 1, 12, 14, and 17 are amended herein to specify that the suppression of demyelination by NGF in the invention occurs within the central nervous system (CNS) of the human or nonhuman primate. Support for such change is replete throughout the application as filed. For example, page 12, first paragraph; page 16, last paragraph; page 17 last paragraph, etc. Because the change introduces no new matter, Applicants respectfully request its entry.

35 U.S.C. §103(a).

Claims 1, 3-6, 12, 13, 17, 19-23, and 25 were rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Kramer *et al.* (*Nature Medicine*, 1995, 1(11):1162-1166) in view of Urschel *et al.* (*Journal of Comparative Neurology*, 1990, 296:114-122), Althaus (WO 9303140), Unger *et al.* (EP 731,108) and Unger *et al.* (1995, Poster: 25th Annual Meeting Society for Neuroscience, San Diego, CA, USA November 11-16, 1995) and in further view of Weiner *et al.* (USPN 5,935,577). Applicants respectfully traverse.

Applicants again submit that the cited references fail to meet *prima facie* obviousness because not all claim elements are present in the combined references. Furthermore, there is

no motivation or suggestion to combine the references and there is no expectation of success in combining them. Thus, even assuming, *arguendo*, that the references could supply all the necessary claim elements, *prima facie* obviousness is still not present since there is no motivation/suggestion to combine the references or expectation of success in doing so.

As stated previously, *prima facie* obviousness from combined references requires that the combination of the cited art, taken with general knowledge in the field, must supply all of the elements of the claimed invention. M.P.E.P. §2143.03. Additionally, there must be a motivation or suggestion to modify the reference(s) or combine the teachings to produce the claimed invention. M.P.E.P. §2143.01 and *In re Geiger*, 815 2 USPQ2d 1276, 1278 (Fed. Cir. 1987). Furthermore, there must be a reasonable expectation of success. M.P.E.P. §2143.02 and *In re Vaeck*, 20 USPQ2d 1438 (Fed. Cir. 1991), citing *In re Dow Chemical Co.*, 5 USPQ2d 1529, 1531 (Fed Cir. 1988). The required teaching or suggestion to combine the references, and the expectation of success, must be found in the prior art and not based upon the disclosure of the Applicants. M.P.E.P. §2142.

Again, Applicants respectfully point out that these requirements have not been met for a *prima facie* showing of obviousness for combinations of the cited references.

The current invention showed the surprising result that treatment of EAE marmosets with NGF suppressed demyelination in the CNS. Work by, *e.g.*, Althaus and Unger, *supra*, had examined *in vitro* studies of NGF and the effect of the use of NGF after chemical injury by a detergent, a condition that spontaneously reverses itself rapidly, and demonstrated that remyelination is accelerated by NGF. However, such findings did not address the more complex and different situation wherein demyelination is caused by inflammation and antibodies, as occurs in MS, a chronic inflammatory condition with multiple immune attacks (to which the EAE marmoset model is similar) and does not occur to a full extent as in the chemical model.

In fact, it would rather be expected that when the remyelination process takes place in MS, unlike in the chemical model, the synthesis and production of new myelin antigens would exacerbate the immune reaction against brain and CNS. However, Applicants' work has demonstrated that in addition to the repair effect shown, NGF suppresses inflammation by a direct effect on astrocytes, suppression of IL-10. This effect is specific for the CNS, as it is not observed in blood (*see* Genain and Villoslada, *Progress in Brain Research*, 2003,

146:403-414). It was surprising and could not have been predicted by those of skill in the art that NGF would inhibit the production of EAE itself and inflammation in the CNS by the second effect, induction of IL-10 locally. In fact, those of skill would more likely have predicted that disease would have developed in the marmoset after induction of EAE, and, as per Unger, then repaired itself faster or more completely in the NGF-treated animals. That was not the observation, as animals failed to fully develop the EAE to begin with. Despite failing to develop EAE, animals had the normal and expected immunological responses against the immunizing antigen, MOG, as measured in blood in the published studies. Thus as would have been expected by those of skill in the art, the marmosets should have developed full-blown EAE, however because of the surprising demonstrated effect of NGF on the astrocytes and the IL-10 production within the CNS, they did not do so. There is no prior art that shows this surprising direct effect of NGF on astrocytes. There is no prior art that shows that this surprising effect of NGF is specific for astrocytes, and it does not occur in peripheral blood cells and lymphocytes. In that, the current results are clearly dissociated from that of Weiner, which only cites ways to modulate peripheral immune system, not glial cells or astrocytes in brain.

Lack of Motivation to Combine

The Office Action bases most of its purported finding of motivation to combine the references on the alleged closeness at the “molecular level” between demyelination and remyelination in demyelination diseases (e.g., MS). The Office Action cites to Unger for support that it would be obvious to combine the cited references. However, again, Applicants respectfully disagree with the Office Action’s reading of the references and of the Office Action’s view of the underlying bases of remyelination/demyelination.

The Office Action cites to Unger (EP 731,108) col. 1, lines 10-23, which states that:

[t]he covering of nerve fibers in the central nervous system (CNS) with myelin is essential for the function of neuronal signal transmission. The myelin sheath is formed by oligodendrocytes (OL), the fibers of which wrap around the axon of a nerve cell. Demyelinating diseases such as multiple sclerosis in which the myelin sheath of the axon is damaged or destroyed also lead to impairments of the OL. However, the OL remains capable of regenerating the myelin sheaths. Therefore, the identification and characterization of factors which are responsible for increased regeneration of OL is very important for the molecular understanding of demyelination diseases, such as multiple sclerosis (MS), and for the development of therapeutic

agents.

Based on the cited paragraph, the Office Action states that the OL is “affected by demyelination and remyelination processes,” and that “identification of factors that affect remyelination are closely related on ‘a molecular level’ to demyelination diseases.” The Office Action also states that Unger links the “two processes” on a “molecular level.” Thus, the Office Action alleges, a skilled artisan would have realized from Unger that “the processes of demyelination and remyelination affect the same substrate, OL, and that the characterization of factors for remyelination are likely to have a similar bases with the factors that lead to demyelination.” The Office Action also states that “demyelination and remyelination processes are closely related” enough so that an “ordinary artisan would have expected that results in one area would provide relevant information for a related area of research” and that such artisan “would have naturally viewed information that reported on myelinating processes as relevant to the field of the instant invention.”

Again, Applicants respectfully disagree with the conclusions drawn from the quotes from Unger and with the Office Action’s assumptions concerning the “closeness” of remyelination/demyelination.

Unger states that “the identification and characterization of factors which are responsible for increased regeneration of OL is very important for the molecular understanding of demyelination diseases.” However, such quote indicates that factors involved in remyelination (e.g., NGF) can be important in understanding (at least a part of) the molecular basis of the disease, it does not state that factors involved in remyelination are important in understanding the molecular basis of demyelination or suppression of demyelination. It doesn’t necessarily follow that factors involved in the disease as a whole or one area of a disease, remyelination, can necessarily be equated with factors involved with the demyelination or suppression of demyelination.

While Unger states that “OL is a significant portion of the myelin sheath and that OL is affected by demyelination and remyelination processes of a nerve,” it does not mention suppression of demyelination. Thus, when the Office Action states that “[c]learly, the identification of factors that affect remyelination are closely related on ‘a molecular level’ to

demyelination diseases,” such statement even if it were true, does not address suppression of demyelination rather than demyelination diseases in general.

Furthermore, while the Office Action states that “the processes of demyelination and remyelination affect the same substrate,” Applicants respectfully point out that it does not necessarily follow from such that “the characterization of factors for remyelination are likely to have a similar basis with the factors that lead to demyelination” let alone with the factors involved with suppression of demyelination. Also as explained below, demyelination and remyelination are actually quite different.

Applicants again point out that understanding of a complex disease such as MS involves multiple facets and areas (*e.g.*, oligodendrocytes and their actions, myelin (as separate from oligodendrocytes), etc.). Thus, a statement that demyelination and remyelination are closely related and therefore that there would be motivation to combine references concerning such to obviate the current invention which concerns suppression of demyelination, is an overly broad reading of the cited quotes and of the field in general.

In fact, the processes of demyelination and remyelination actually do not affect the same substrate, and are not closely related, although they involve the same cell, OL, in the CNS. Demyelination is an external aggression on OL and myelin sheaths, for example by inflammation, stroke, trauma, chemical, infectious, or other external circumstance causing injury to OL. Remyelination is an intrinsic molecular process that involves many unrelated and complex stimuli, such as growth factors, signaling between nerve axons and myelin, etc. There is little relationship between the two processes at the molecular level, one being external aggression and the other being internal to the brain and CNS environment. This is exemplified by the finding that in disorders like MS and the EAE model, remyelinating cells are present at the periphery of the demyelinating lesions but are not capable of providing remyelination. *See, e.g., Wolswijk, et al., Brain, 2002, 125:228-349.* Remyelination in addition, does not occur in MS to a full extent after the demyelination has occurred.

As illustrated by the discussion above comparing the surprising *in vivo* results against prior *in vitro* studies, it can be seen that there would be no motivation to combine such studies to achieve the current invention.

Again, as discussed the demonstration of effect in *in vitro* culture systems was inadequate to predict the effects *in vivo* and demonstration of efficacy in a chemical model or

the like (*e.g.*, where demyelination is caused by detergent) could not be extrapolated to efficacy in a model such as EAE or MS where there is persistent inflammation driven by a chronic immune response against myelin and nerve cells. Furthermore additional differences between demyelination and remyelination (emphasizing the lack of motivation in combining such references) include that the cells that provide remyelination are derived from precursor cells, not from mature OL. The action of NGF facilitates this transformation. Studies of NGF or other growth factors and neurotrophins conducted on neonatal or young OL are not always relevant to biology of adult OLs, as exemplified by the fact that neonatal microglia are exquisitely sensitive to injury, *e.g.*, by oxidative radicals, while adult glia are resistant to such. Furthermore, with regard to inter-species differences, rodent microglia and primate/human microglia have very different biologies, especially with respect to expression of constitutive and inducible NOS genes which regulate pathways of injury to these cells. *See below* for other problems in comparing results from different species.

In regard to Kramer, *supra*, there also would be little motivation to combine its teachings with the other references to achieve the current invention because it examines EAN, a model of injury of the peripheral nervous system (PNS), while MS and EAE are diseases of the central nervous system (CNS). The natural barriers between PNS and blood, and CNS and blood, are of very different nature. For example, the cells that provide remyelination (repair) in PNS are Schwann cells, whereas in CNS they are OL.

Thus, because of the differences in demyelination and remyelination there would be no motivation to combine the cited references. Therefore because there is no motivation to combine the references (and because of the surprising results), Applicants respectfully request that the rejections be withdrawn.

Applicants also emphasize again that even if the Office Action's statement that "the ordinary artisan would have expected that the results in one area concomitantly provide relevant information for a related area of research" were true, it implies that it is an invitation to experiment, which is not a legitimate basis for establishing obviousness. *See, e.g., Ex Parte Erlich*, 3 USPQ2d 1529 (Fed. Cir. 1988); *In re Geiger*, 2 USPQ2d 1276 (Fed. Cir. 1987); *In Re Dow*, 5 USPQ2d 1529 (Fed. Cir. 1988); and *In Re Eli Lilly & Co.* 14 USPQ2d 1741, 1743 (Fed. Cir. 1990).

Lack of Expectation of Success

The cited references also fail to present *prima facie* obviousness because there is no reasonable expectation of success, fully achieving the desired invention, in combining the references. As previously stated, even if Kramer, *arguendo*, contains statements concerning decreasing demyelination in peripheral rodent neurons, there is no expectation of success in combining it with the rat model in Urschel and the porcine/human in Althaus, etc., to achieve the current invention (human/primate). Again, as pointed out in the specification at page 19,

It is important to recognize that, due to the interspecies differences in the biological effects of growth factors and the differential expression of their specific receptors, information derived from rodent studies may not be applicable to humans.

Such passage highlights the lack of expectation of success for combination of the cited references. The Office Action cites to Weiner *et al.* (USPN 5,935,577) for support that there was an expectation of success in combining the various animal models of the many references cited. The Office Action also argues that Applicants do not present data that specifically rebuts Weiner's alleged teaching that EAE animal models are good approximation for responses in humans or present any specific examples as to why the statements by Weiner are invalid.

In addition to the passage from the present application, numerous references specifically emphasize the failings of using rodent models for developing treatments for MS and the inadequacies in translating rodent results to primates/humans (specifically in regard to EAE).

For example, in comparing rodent EAE models with common marmoset models, 't Hart states that:

[I]n many cases, therapies based on biological reagents are highly species-specific. This implies that reagents developed for treatment of humans should be tested in nonhuman primates. Of the wide variety of immuno-suppressive/modulatory therapies that were tested in MS after proven effectivity in EAE models, only very few have shown beneficial effect in patients.

't Hart *et al.*, *Curr. Op. Neuro.*, 2003, 16:375-383, 379.

Even more bluntly, in discussing animal models for MS, 't Hart states that "[t]he wide immunological gap between human beings and laboratory mouse or rat models makes many disease models in these species invalid." 't Hart *et al.*, *Lancet Neurol*, 2004, 3:588-597,

abstract. Additionally, 't Hart states that "[c]urrent disease models in rodents might not be sufficiently predictive of therapeutic success in humans, a situation that is not unique for MS." 't Hart, *et al.*, *Trends in Molecular Medicine*, 2004, 10(2):85-91.

Thus, the effects of an NGF (e.g., a recombinant human NGF) molecule, a protein and biological with species-specificity, can only be truly appreciated and tested in a system such as the primate, that approximates humans and expresses a reasonably homologous receptor.

Weiner presents an EAE model in the Lewis rat that is induced with immunization with MBP. Such model is characterized by the absence of demyelination and a monophasic course, which may be an adequate model for inflammation of the brain but is an inadequate model for MS which has demyelination and a relapsing course. MBP similarly does not induce demyelination in marmosets (*see Genain et al.*, *J. Clin. Invest.*, 1995, 96:2966-2974), neither do T cells directed against MBP, which are sufficient to produce CNS inflammation but not demyelination (*see Genain et al.*, *J. Clin. Invest.*, 1994, 94:1339-1345). The demyelination in the marmoset model and MS has a different basis, antibody and B cell mediated toxicity than the cited references. *See, e.g.*, Raine *et al.*, *Annals of Neurology*, 1999; 46(2):141-2 and Genain, *et al.*, *Nat Med.*, 1999, 5(2):170-175. This illustrates that, the animal models from different species (such as the rats in Weiner) have different responses to specific substances, etc., and thus there would not be an expectation of success in combining them.

Yet another example of the inadequacy of rodent models such as Weiner's to predict efficacy for treatment of MS is shown by the failure in human trials of TGF-beta, and TNF inhibition. *See Arnason, et al.*, *Neurology*, 1999: 53:457-65 (also even including the possibility that anti-TNF therapy triggers MS, Sicotte *et al.*, *Neurology*, 2001, 57:1885-1888).

Further support for the argument that inter-species differences are critical, especially in view of neural growth factors, is shown by Aredondo, *Eur. J. Immunol.*, 2001, 31:625-633, which shows that NGF-based intervention in rodents may have adverse effects, or may not work on amelioration of disease. A compelling argument is also that NGF produces radically different effects on rat oligodendrocytes, which it kills through the p75 receptor pathway

(Casaccia-Bonnet, *et al.*, 1996, *Nature*, 24:383(6602):716-9), and human oligodendrocytes (Ladiwala, *et al.*, 1998, *J. of Neurosci.*, 18:1297-1304).

An example of a broader discussion of the inadequacy of rodent models to predict human disease behavior, as exemplified by the failure of such models to predict efficacy of an Alzheimer's vaccine, is shown in, *e.g.*, Mestas, *et al.*, "Of Mice and Not Men: Differences between Mouse and Human Immunology," *J. Immunol.*, 2004, 172:2731-2738.

Thus, all such examples illustrate that results are not reasonably translated between species in MS studies. Therefore, EAE rodent studies are not easily extrapolated to MS in primates and humans. Applicants submit that there would be no reasonable expectation of success fully achieving the current invention in combining data from the various animal models in the numerous references cited.

Other aspects of the cited references also highlight why no expectation of success exists in their combination. For example, Kramer allegedly concerns decreased demyelination in peripheral rat neurons, while Althaus focuses on regeneration of myelin and Urschel focuses on showing that removal of NGF affects myelination (not that adding NGF increases myelin). As detailed above, Applicants respectfully point out that the references concern different actions/aspects of myelin (*e.g.*, regeneration of myelin rather than demyelination, decreased demyelination, etc.) and that such areas are quite different in terms of causation, mode of action, etc., and therefore, there would be no reasonable expectation of success in combining them.

Because there is no reasonable expectation of success fully achieving the current invention in combining the references, Applicants respectfully request that the rejections be withdrawn.

Individual References

The Office Action states that Applicants are attacking the references individually by not considering their common elements. However, Applicants are merely pointing out how the elements are not common between the references and how the large underlying differences between the animal models and specific actions studied vary between the references, and thus would not favor motivation to combine.

Again, Applicants note that the references are not being argued against individually. Rather, by stating the differences between the references as a group, it is emphasized how

they each concentrate on different areas and how results from one area are not reasonably transferred to another and, thus, how there would be no expectation of success, fully achieving the desired invention, in combining the references.

Lack of all Claim Elements

In addition to lack of a motivation to combine the references and an expectation of success in doing so, the combinations of the cited references do not even present all the elements of all of the current claims. For example, the cited references do not include NGF (as used in claims 17-25) in an amount sufficient to downregulate the production of interferon γ (IFN- γ) by T cells infiltrating the central nervous system. The Office Action cites to Weiner and argues that it demonstrates that successful treatment of MS (such as by suppressing demyelination with NGF) will inherently down regulate the production of IFN- γ by T cells.

The fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. M.P.E.P. §2112(IV) citing *In re Rijckaert*, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993). To establish inherency, the extrinsic evidence must make clear that the missing descriptive matter is necessarily present in the reference. Inherency cannot be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient. M.P.E.P. §2112(IV) citing *In re Robertson* 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999).

Applicants note that their prior argument was not necessarily that NGF would not inherently down regulate production of IFN- γ by T cells, but rather that such case had not been made by the Office Action.

Applicants submit that the cited passages in Wiener do not present *prima facie* inherency. For example, the cited passages in Weiner do not even concern the same subject as the current invention. The discussion of IFN- γ levels in col. 9, lines 25-34 concerns their use to check the efficacy of mucosally induced bystander suppression.

Weiner also does not show that NGF suppresses IFN- γ from within the brain/CNS. Werner does not demonstrate the increased production of IL-10 by astrocytes, rather than lymphocytes, after stimulation with NGF. As exemplified by Cannella (*J. Neurosci. Res.*, 1996, 45(6):735-46) and Cua (*J. Exp. Med.*, 1999, 189(6):1005-1010), production of IL-10

within the CNS, and not in the periphery, which is achieved by NGF because of its specific effects on astrocytes, is the driving force for IFN- γ suppression and disease inhibition (inflammation). This effect is completely independent of the remyelination processes, as discussed above. Applicants also note that this point further argues that there is no reason for combining the previous art cited.

Weiner does not mention NGF and does not teach that NGF reduces IFN- γ in peripheral lymphocytes (let alone whether such is inherent in successful treatment) because Weiner did not study such. Weiner does not teach that NGF reduces IFN- γ or induces IL-10 in the central nervous system. Weiner does not claim that suppression of IFN- γ in CNS will suppress autoimmunity,

Finally, and quite on point, the successful treatment of autoimmune disease will not always require a decrease in IFN production. This is shown by the fact that mice who do not produce IFN- γ can have worse EAE disease. Even more striking, is the finding that Th1 responses mediated by IFN- γ and TNF may actually benefit repair and remyelination in CNS, as shown by work on crushed optic nerve in rodent and EAE (Kipnis, *et al.*, *J Neuroimmunol*, 2002, 130:78-85; Kipnis, *et al.*, *PNAS*, 2000, 97(13):7446-6451; Moalem, *et al.*, *FASEB*, 1999, 13:1207-1217; Hohlfeld, *et al.*, *J. Neuroimmunol.*, 2000, 170:161-166).

Thus, not only does Weiner examine dissimilar material than the current invention (thus emphasizing that the required claim elements are not present either explicitly or inherently), additional references cite to instances wherein IFN- γ will not always necessarily need to be reduced in the treatment of an autoimmune disease. Therefore, Applicants respectfully request that the rejection be withdrawn.

In sum, the cited references fail to meet the three required aspects of *prima facie* obviousness because there is no motivation/suggestion to combine the references, there is no expectation of success in combining the references, and not all elements of all of the current claims are present in the references. Therefore, Applicants respectfully request that the rejections be withdrawn.

Claims 14 and 15 were rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Kramer *et al.* (*supra*) in view of Urschel *et al.* (*supra*), Althaus (WO 9303140), Unger

et al. (EP 731,108) and Unger *et al.* (*supra*) and in further view of Hammang *et al.* (USPN 5,904,144) and The Merck Manual (p. 1091). Applicants respectfully traverse.

As illustrated above, there is no motivation/suggestion to combine, nor expectation of success in combining Kramer, Urschel, Althaus, Unger, and Unger. Because there is no underlying motivation/expectation tying Kramer, Urschel, Althaus, Unger, and Unger together, there is no motivation/expectation of success to add Hammang or Merck. In other words, the combination of references against claims 14 and 15 fails for similar reasons as stated above.

Thus, the cited references fail to meet the required aspects of *prima facie* obviousness because, *e.g.*, there is no motivation/suggestion to combine the references, and there is no expectation of success in combining the references. Therefore, Applicants respectfully request that the rejections be withdrawn.

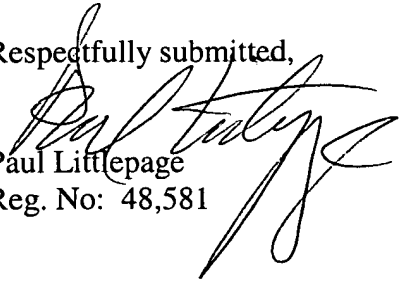
CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the claims are deemed not to be in condition for allowance after consideration of this Response, please telephone the undersigned at (510) 769-3507.

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Role of nerve growth factor and other trophic factors in brain inflammation

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Abstract: Inflammation in the brain is a double-edged process that may be beneficial in promoting homeostasis and repair, but can also result in tissue injury through the damaging potential of inflammatory mediators. Thus, control mechanisms that minimize the extent of the inflammatory reaction are necessary in order to help preserve brain architecture and restore function. The expression of neurotrophic factors such as nerve growth factor (NGF) is increased after brain injury, in part mediated by effects on astrocytes of pro-inflammatory mediators and cytokines produced by immune cells. Conversely, cells of the immune system express NGF receptors, and NGF signaling modulates immune function. Multiple sclerosis (MS) and the disease model experimental autoimmune encephalomyelitis are neurodegenerative disorders whereby chronic destruction of the brain parenchyma results from an autoaggressive, immune-mediated inflammatory process and insufficient tissue regeneration. Here, we review evidence indicating that the increased production of NGF and other trophic factors in central nervous system (CNS) during these diseases can suppress inflammation by switching the immune response to an anti-inflammatory, suppressive mode in a brain-specific environment. Thus, trophic factors networks in the adult CNS not only protects axons and myelin but appear to also actively contribute to the maintenance of the brain immune privilege. These agents may represent good targets for therapeutic intervention in MS and other chronic CNS inflammatory diseases.

Keywords: neural growth factors; autoimmune demyelination; multiple sclerosis; experimental autoimmune encephalomyelitis; cytokines; immunomodulation

Introduction

In addition to its the critical role during central nervous system (CNS) development, nerve growth factor (NGF) plays an important role in the maintenance of adult CNS homeostasis and in the response to brain tissue damage (Kernie and Parada, 2000). Due to the highly specialized architecture and function of the CNS, maintenance of brain tissue

integrity of it is fundamental for the survival of the individual. Bystander effects of inflammation, although well tolerated in most tissues such as epithelium or connective tissues, might be deleterious when occurring within the CNS. It has been proposed that the CNS is protected against such adverse consequences of inflammation through immune privilege (Streilein, 1995; Antel and Owens, 1999), which prevents invasion of brain parenchyma by inflammatory cells and/or partially restricts local immune responses by maintaining an immunosuppressive environment. In this short review we will focus on the roles of NGF during CNS inflammation and on newly discovered immunoregulatory properties of NGF and other neural growth factors that are

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likely to contribute to the maintenance of CNS immune privilege.

During pathological situations such as CNS infections or autoimmune diseases, an inflammatory response is mounted against the CNS that may result in 'bystander' tissue destruction and loss of function. Multiple sclerosis (MS) is the prototypal inflammatory brain disease of probable autoimmune origin, in which infiltrating inflammatory immune cells and mediators contribute to CNS damage and lead to widespread destruction of the myelin sheaths, oligodendrocytes and axons, and gliosis (Steinman, 2001). The chronic accumulation of such lesions along the neuraxis profoundly disturbs nerve conduction and leads to significant disability, often in young adults. Autoimmune demyelination is a good example of disorders in which inflammation is detrimental to the CNS, which affords to the study of the complex interplay between inflammation, immune privilege, tissue destruction, and mechanisms of repair and neuroprotection.

NGF and immune system interactions

Several immune cell types express the NGF receptors TrkA and p75, and it has been demonstrated that NGF production is increased in inflammatory diseases. The role of NGF in the regulation of immune response is complex and not fully understood (Aloe et al., 1994; Levi-Montalcini et al., 1996). For example, NGF production is induced in brain cells by a variety of pro-inflammatory and anti-inflammatory cytokines such as interleukin-1 (IL-1), IL-4, IL-5, tumor necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β) and interferon- β (IFN- β) mediated by NF κ B signaling (Gadient et al., 1990; Awatsuji et al., 1993; Awatsuji et al., 1995; Friedman et al., 1996; Boutros et al., 1997). It is also of importance to keep in mind that the effects of NGF on inflammation may depend on the microenvironment, and immunomodulatory properties of NGF in the brain may be different from those in other tissues.

The TrkA receptor and p75 neurotrophin receptor (p75^{NTR}) are expressed both in cells of

the innate immune system, especially in mast cells and macrophages, and in adaptive immune system cells, such as T cells (Ehrhard et al., 1993; Santambrogio et al., 1994) and B cells (Brodie and Gelfand, 1992; Melamed et al., 1996). NGF signaling may promote survival of memory B cells (Torcia et al., 1996), influences T cell and B cell proliferation, stimulates the synthesis of immunoglobulins (IgM, IgA and IgG4) (Brodie, 1996). The role of NGF in the innate immune system is also complex. Mast cells are most responsive to NGF, and themselves release NGF in an autocrine manner in response to inflammatory stimuli. Stimulation of mast cells by NGF promotes survival, induces the secretion of cytokines such as IL-3, IL-4, IL-10, TNF- α and granulocyte macrophage colony-stimulating factor (GM-CSF), and modifies vascular permeability (Bullock and Johnson, 1996). NGF also influences differentiation of monocytes into macrophages, enhances phagocytosis and increases antimicrobial activity. Lipopolysaccharide (LPS), a pathogen product, induces the release of NGF and the expression of NGF receptors by macrophages (Caroleo et al., 2001). Together these findings provide evidence that NGF plays a complex role in the regulation of inflammatory responses, with specific effects that may vary depending on the organ or tissue involved.

Because of the existence of a blood-brain barrier (BBB) that ensures its immunologically privileged situation, immune responses in the CNS are mainly mediated by resident microglia, mast cells and astrocytes. However, in CNS inflammation the BBB is disrupted and immune mediators from the peripheral immune system may enter the parenchyma, which results in a fully developed adaptive immune response with loss of immune privilege. Perivascular mast cells and macrophages are highly sensitive to NGF, and it has been postulated that this interaction might contribute to the integrity of BBB and its restoration after CNS inflammation (Ransohoff and Trebst, 2000; Flugel et al., 2001). In adult CNS, NGF is mainly produced by astrocytes and stored into the extracellular matrix (ECM). The ECM represents a reservoir of trophic factors that interact with immune cells entering the CNS to modulate the ongoing immune response.

Role of NGF in CNS autoimmune demyelination

Experimental autoimmune encephalomyelitis (EAE) is a widely used disease model for MS of autoimmune pathogenesis (Bradl and Linington, 1996). In EAE, activated self-reacting CD4+ and CD8+ T cells, macrophages, and anti-myelin antibodies invade the CNS and mediate tissue damage either directly or through a variety of effector mechanisms such as production of nitric oxide, free radicals, and activation of complement or macrophage mediated cytotoxicity (Brosnan and Raine, 1996). Myelin sheaths and oligodendrocytes are the primary targets of the autoimmune attack, and axonal transection and gliosis are also present. During EAE, expression of NGF and NGF receptors is increased in astrocytes, oligodendrocytes and in cells from the subventricular zone (De Simone et al., 1996; Calza et al., 1998; Micera et al., 1998; Oderfeld-Nowak et al., 2001). The increased expression of NGF parallels that of a number of other neurotrophic growth factors, and may represent as in other forms of CNS tissue injury, a regulatory mechanisms aimed at promoting tissue repair. Another interesting possibility is that growth factors influence development and severity of autoimmune inflammatory demyelination, as suggested by reports of decreased severity after administration of insulin growth factor (IGF)-1, glial growth factor (GGF)-2, ciliary neurotrophic factor (CNTF) and NGF in rodent EAE models (de Webster, 1997; Cannella et al., 1998; Lovett-Racke et al., 1998; Arredondo et al., 2001; Linker et al., 2002), and aggravation upon administration of a neutralizing NGF antibody (Micera et al., 2000).

We recently evaluated the efficacy of recombinant human NGF using the marmoset model of EAE, which offers close phenotypic similarity with human MS including a relapsing remitting clinical course and pathologically, moderate perivascular inflammation accompanied by prominent demyelination at the acute stage (Genain and Hauser, 2001). Animals were treated intracerebroventricularly with 6 µg per day of recombinant human NGF or placebo, starting one week after immunization (typically one week before the onset of CNS infiltration) until two weeks after the onset of the clinical disease (first relapse). The original trial was designed in order to probe for

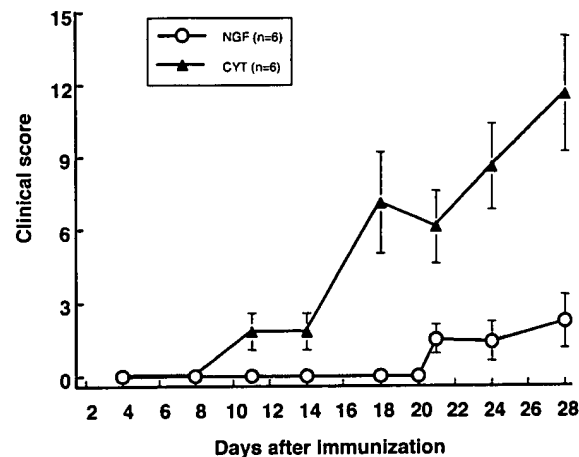


Fig. 1. Recombinant human NGF inhibits the development of clinical EAE in marmosets. EAE was induced by active immunization with myelin/oligodendrocyte glycoprotein in adjuvant, which in this species results in 100% incidence of disease. NGF (open circles) or placebo (cytochrome c, CYT, closed triangles) were administered intracerebroventricularly by continuous infusion (osmotic pump) beginning day 7 after immunization. EAE was graded using an expanded marmoset grading scale ranging from 0 to 45. Neuropathological findings throughout the CNS (optic nerves, brain and spinal cord) corroborated the observed clinical protection. * $p < 0.05$ student's *t*-test. For details please refer to Villoslada et al. (2000).

improved remyelination due to NGF therapy. However, clinical and neuropathological features of EAE were milder in NGF-treated animals than in placebo-treated controls (Fig. 1). Immunohistochemical studies demonstrated that CNS infiltrating mononuclear cells of the NGF-treated animals expressed lower amounts of interferon-gamma (IFN- γ), the prototypical Th1 cytokine in EAE, and higher amounts of IL-10, a major immunosuppressive cytokine, which indicated that the CNS-specific immune response had been induced to a switch from the proinflammatory Th1 phenotype to an anti-inflammatory Th2 phenotype. Perhaps more strikingly, glial cells within the unaffected white matter expressed higher amounts of IL-10 even in areas remote from inflammatory infiltrates (Figs. 2 and 3). The latter finding suggests that NGF had the ability to induce a CNS-wide immunosuppressive microenvironment that may have been the primary mechanism leading to decreased CNS infiltration, reduction in BBB breakdown and IFN- γ suppression

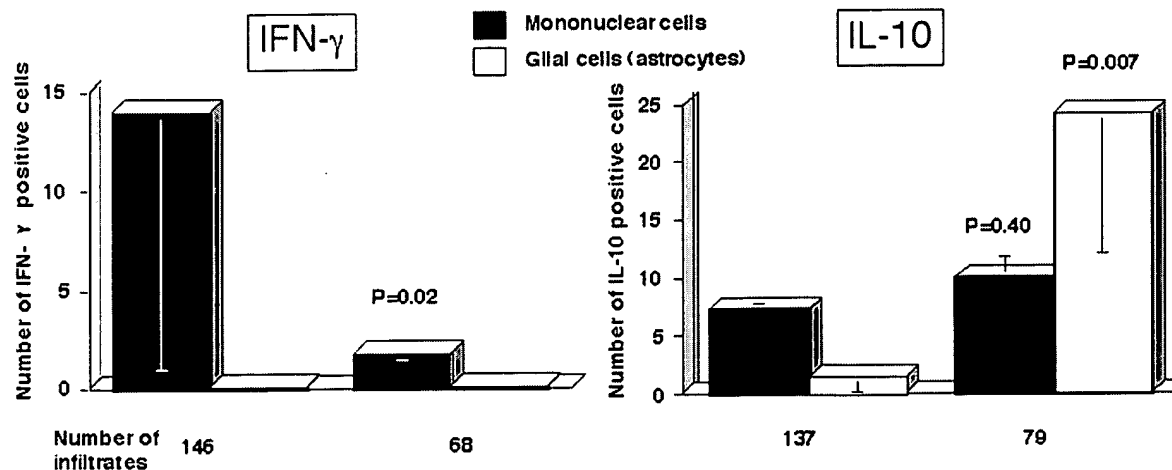


Fig. 2. Effects of recombinant human NGF on cytokine production (IFN- γ and IL-10) within CNS inflammatory infiltrates during marmoset EAE.

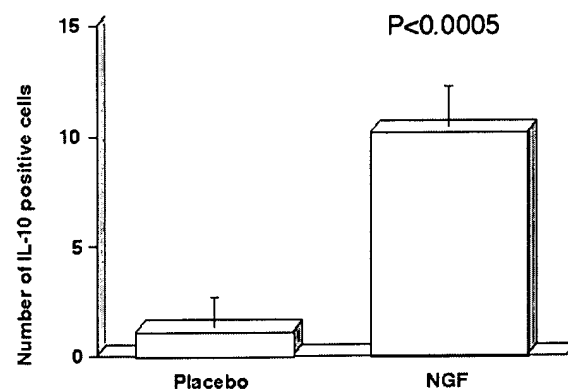


Fig. 3. Effects of recombinant human NGF upon IL-10 production by glial cells of normal, unaffected white matter during marmoset EAE. A striking upregulation of IL-10 is observed in areas at distance from the pathological infiltrates, suggesting that intraventricular administration of NGF induced a tissue-specific immune deviation that suppressed CNS inflammation mediated by IFN- γ -producing inflammatory infiltrating cells.

in treated animals (for details see Ransohoff and Trebst, 2000; Villoslada et al., 2000). These CNS-specific immunosuppressive properties of IL-10 may be specific for higher mammal species (human and nonhuman primates), since they have not been unequivocally demonstrated in rodents (Cannella et al., 1996; Bettelli et al., 1998; Cua et al., 1999).

The pronounced effects of intracranial administration of NGF on cytokine expression were specific to the CNS parenchyma, and were not observed in the periphery. T cell-mediated EAE in rodents can classically be inhibited by promoting an immune bias of myelin-reactive T cells towards the Th2 phenotype (immune deviation), or by administration of Th2 cytokines (Chen et al., 1994; Racke et al., 1994). This approach has yet to prove useful in human clinical trials, and immune deviation of the peripheral immune system may trigger lethal complications in CNS demyelinating disorders of complex pathophysiology because it has the potential to exacerbate pathogenic antibody responses (Genain et al., 1996). Peripheral administration NGF could also potentially exacerbate such responses, since NGF is a factor that promotes B cell survival and growth (Aloe et al., 1994; Levi-Montalcini et al., 1996; Torcia et al., 1996). However, intracranial administration of NGF in marmosets did not alter the expected development of T cell and humoral autoimmune responses against the immunizing antigen and did not induce immune deviation in the peripheral immune system, confirming that effector mechanisms for the beneficial effects were taking place within the CNS (Villoslada et al., 2000). To further confirm these findings, we evaluated the effect of administration of exogenous NGF on the production of Th1 (TNF- α , IFN- γ and IL-12) and Th2 (IL-4, IL-10, IL-6 and TGF- β)

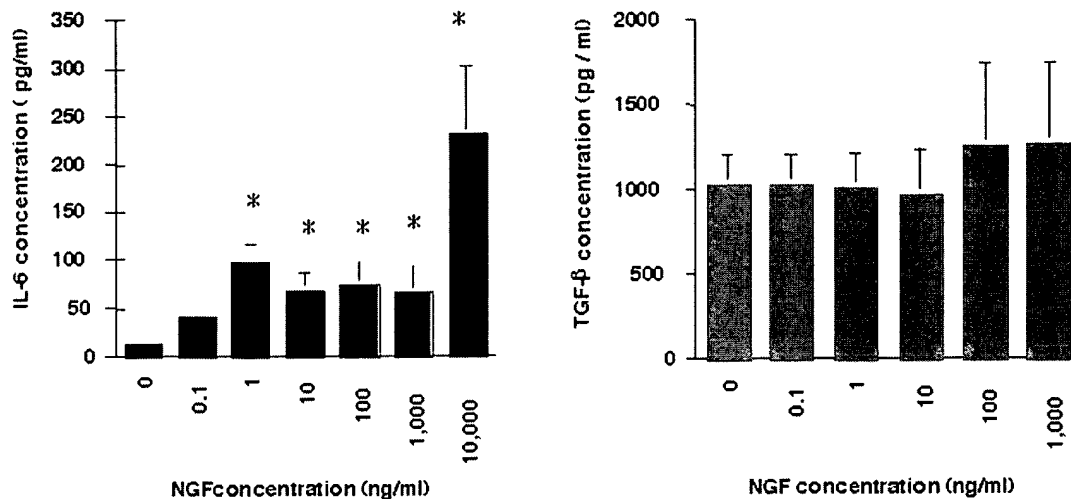


Fig. 4. Effects of exogenous recombinant human NGF on lectin-induced secretion of cytokines by PBMC. The only significant change detected was an increased secretion of IL-6. Please refer to the text for details of cytokines examined.

cytokines by cultured peripheral blood mononuclear cells (PBMC) and macrophages stimulated *in vitro* by antigen or lectins. In both human and marmoset systems, all cytokines examined remained unaffected by addition of NGF, except for IL-6 which was markedly increased with a dose-dependent response (Fig. 4). This additional effect of NGF on IL-6 production by peripheral blood mononuclear cells (PBMC) (which was not examined in the CNS of NGF-treated marmosets), is of interest since this cytokine has been shown to suppress disease both in EAE (Willenborg et al., 1995; Carr and Campbell, 1999) and in a viral experimental model of MS (Rodriguez et al., 1994). Nonetheless, the upregulation of IL-10 in CNS glial cells (and not PBMC) during treatment of marmosets with EAE highlights a unique property of NGF in inducing a form of tissue-specific immune deviation that is capable of modulating CNS autoaggressive responses and likely contributed to the maintenance of the CNS immune privilege.

The immunosuppressive properties of NGF in EAE were confirmed in a mouse model carrying a transgene encoding for the T cell receptor specific for an encephalitogenic epitope of myelin basic protein. NGF was expressed by T cells in response to exposure to a Th2 environment (IL-4), but *in vitro* administration of NGF did not change Th phenotypes or proliferation responses. Intraperitoneal

administration of NGF during the induction phase of EAE ameliorated the disease without changing the encephalitogenic properties of the T cells. The interpretation of these results was that NGF may exert an indirect role in modulating the peripheral immune response, perhaps through enhanced sympathetic innervation of lymphoid tissue (Arredondo et al., 2001). A regulatory role for anti-inflammatory properties of NGF is also supported by the demonstration that antigen-specific Th1 cells engineered to deliver NGF *in situ* ameliorate disease and inhibit transmigration of inflammatory cells in several models of CNS or peripheral nervous system autoimmune demyelination (Kramer et al., 1995; Flugel et al., 2001). What mediates these anti-inflammatory properties in addition to, or via the secretion of suppressive cytokines such as IL-10 is not entirely understood, but may involve the down-regulation of the expression of major histocompatibility complex (MHC) molecules on the resident CNS antigen presenting cells, which in EAE function by reactivating invading antigen-specific T cells (Frei et al., 1994; Neumann et al., 1998).

In summary, data from EAE models provide enough evidence for a regulatory role of NGF in autoimmune demyelination, due to the overexpression of NGF and NGF receptors during CNS inflammation and *in vivo* experiments that demonstrate protection from disease. Studies conducted to

date suggest that NGF is secreted both in paracrine and autocrine fashions, and possesses pleiotropic functions that cannot be regarded as classical immunosuppressive activities in lymphocytes such as for the suppressive cytokine IL-10. NGF may work by multiple mechanisms, including maintenance of BBB integrity, modulation of the overall immune system activity through sympathetic innervations, induction of an immunosuppressive microenvironment in situ in the CNS, and survival and restoration enhancement of oligodendrocytes and axons after immune-mediated damage. Finally, it is important to recognize that the relative importance of these different immunomodulatory pathways may vary between species.

Possible role of NGF in multiple sclerosis

In MS lesions, NGF receptors are expressed mainly by inflammatory cells such as microglia and monocytes and to a lesser extent by astrocytes but not by oligodendrocytes (Valdo et al., 2002). However, mRNA^{p75^{NTR}} has been detected at increased levels in oligodendrocytes in MS plaques (Dowling et al., 1999). These findings and reports that oligodendrocytes expressing p75 may undergo apoptosis in vitro (Casaccia-Bonnel et al., 1996; Frade et al., 1996) raises an interesting question, because oligodendrocyte death is a pathological hallmark in multiple sclerosis. By contrast, it has also been shown that p75^{NTR} signaling in stressed oligodendrocytes promotes survival (Lopez-Sanchez and Frade, 2002). The role of NGF during inflammatory demyelination in adult CNS may depend on the relative levels of expression of the two NGF receptors, and remains to be clarified taking into account the fact that the very same infiltrating lymphocytes that mediate parenchymal inflammation may express NGF as well as other neurotrophins such as brain-derived neurotrophic factor (BDNF) (Kerschensteiner et al., 1999; Moalem et al., 2000; Muhallab et al., 2002; Stadelmann et al., 2002). Thus, inflammatory effector cells themselves may, at least in part, support neuroprotective functions through the secretion of trophic factors, perhaps representing a self-limiting mechanism for inflammation that may be deficient in the case of CNS autoimmune damage.

During acute attacks, patients with MS have increased levels of NGF in the CSF compared with healthy controls or patients that are clinically stable, which may be interpreted as evidence that NGF is produced in an attempt to protect CNS tissue against inflammation (Laudiero et al., 1992). We have replicated these findings by assessing the levels of NGF in the CSF of patients with relapsing-remitting (RR) MS during an attack, patients with secondary-progressive (SP) MS, patients with a first manifestation suggestive of a demyelinating event (clinically isolated syndrome, CIS), healthy controls, and patients with other neurological disease than MS (P. Villoslada, unpublished results—Fig. 5). We found that NGF levels were higher in the CSF of patients with MS than in healthy controls or patients with other neurological diseases. Interestingly, NGF levels were on average higher in MS patients with RR-MS that typically display disseminated disease with evidence of active CNS inflammation and BBB breakdown (Gadolinium enhancement on magnetic resonance imaging), compared to patients with SP-MS in which inflammation is typically less prominent, and patients with a CIS who by definition lack evidence of disease dissemination. Together, our interpretation of these data is that NGF is produced during CNS damage as a means to recover brain homeostasis.

Role of other trophic factors in CNS immunosuppression and in neuroprotection

Other trophic factors than NGF clearly share similar immunosuppressive and neuroprotective properties. TGF- β is an immunosuppressive cytokine that is increased in tissues with immune privilege in order to prevent their damage by the immune system, and is able to induce immune deviation (Streilein et al., 1997). TGF- β KO mice develop spontaneous autoimmune diseases involving several organs including the brain (Schull et al., 1992), and treatment of animals induced for EAE with TGF- β prevents the disease (Racke et al., 1991). Insulin-like growth factor-1 (IGF-1) is another pleiotropic growth factor capable to suppress murine EAE when the treatment was started before disease onset, however in one study disease was exacerbated when treatment was

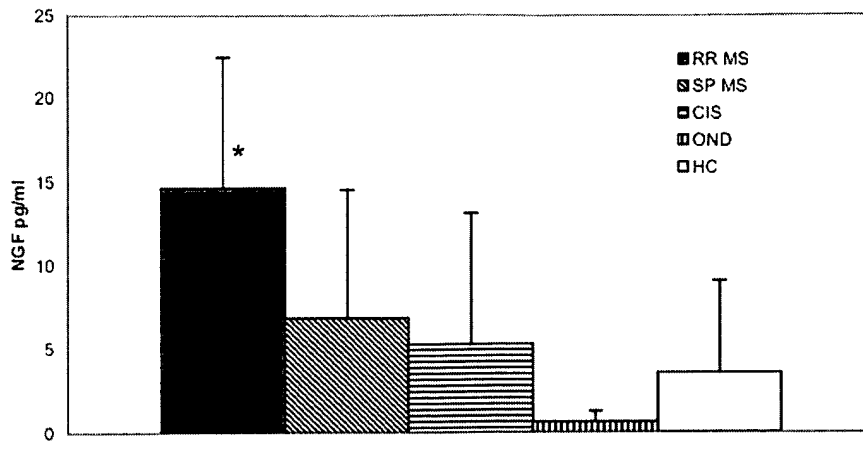


Fig. 5. Levels of NGF in the CSF from patients with relapsing-remitting MS (RR-MS) during a relapse ($n=17$), secondary-progressive MS (SP-MS, $n=16$), clinically isolated syndrome (CIS, $n=16$), other neurological diseases (OND, $n=16$) and healthy controls (HC, $n=16$) from whom CSF was obtained in the differential diagnosis of acute headache. Levels of NGF in RR-MS patients during relapses were significantly higher than in the other groups ($p < 0.0001$, one factor ANOVA).

started after disease onset (de Webster, 1997; Lovett-Racke et al., 1998). Similarly, neuregulin or glial growth factor-2 (GGF-2) administered systemically prevented the development of murine EAE (Cannella et al., 1998). Disease attenuation appeared to be due to both increased remyelination and decreased CNS inflammatory activity which, reminiscent of our studies in marmosets may have been due to enhanced expression of the immunosuppressive cytokine IL-10.

Recently, two additional members of the neurocytokine family leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) were found to protect oligodendrocytes during murine EAE (Butzkueven et al., 2002; Linker et al., 2002). Oligodendrocytes from LIF-treated animals were preserved from inflammatory attack and stressed oligodendrocytes expressed increased levels of LIF receptor- β that might account for the protection against oligodendrocyte loss. However, an immunosuppressive role was not demonstrated although immune cells expressed LIF receptors and treatment was administered systemically. CNTF deficient mice had more severe disease and recovery was poor with a 60% decrease in the number of proliferating oligodendrocyte precursors and myelin vacuolation. Thus, there may be differential contributions of trophic factors to maintenance of the immune privilege of the brain, to neuroprotection and CNS regeneration, and to immunosuppression.

In addition to NGF, we have recently analyzed CSF levels of LIF and glial cell line-derived neurotrophic factor (GDNF) in MS patients. We found CSF levels of LIF and GDNF are higher in patients with active disease than in those with chronic disease or healthy controls, which confirms that NGF is among a number of trophic factors released in response to brain damage (Table 1). These findings further support the role of the trophic factor network in promoting CNS regeneration after inflammatory damage and in maintaining an immunosuppressive microenvironment in the CNS.

Proposed model for trophic factors in CNS immune privilege and neuroprotection

The data reviewed here strongly argue that NGF (and other trophic factors) are capable of suppressing autoaggressive immune responses in a CNS-specific fashion which does not involve modulation of the peripheral immune system, likely indicating that this function can only be accomplished in the context of the CNS microenvironment. The immune privilege of the brain, although not fully understood, can be maintained by the additive effects of physical barriers such as the BBB and the lack of lymphatic vessels in CNS tissue, active downregulation of HLA antigen-presenting molecules (astrocytes and microglia), and

TABLE 1

Levels of trophic factors in serum and CSF from patients with relapsing-remitting MS (RR-MS), primary-progressive MS (PP-MS) and healthy controls (HC)

Trophic factor (pg/ml)	Serum			CSF		
	RR-MS	PP-MS	HC	RR-MS	PP-MS	HC
NGF	ND	ND	ND	14.6 ± 7	6.9 ± 7	3.62 ± 5
LIF	7.9 ± 13	ND	11.6 ± 21	11.7 ± 14	8.4 ± 19	1.7 ± 4
GDNF*	0	0	0	160 ± 10	70 ± 8	40 ± 7

*Total GDNF (after acidification treatment to measure both free and protein bind GDNF).

upregulation of immunosuppressive cytokines such as IL-10 or TGF- β . Another proposed mechanism is the expression of pro-apoptotic molecules such as the Fas-ligand that promote apoptosis of infiltrating activated lymphocytes, although a clear demonstration of such phenomena is still lacking (O'Connell et al., 2001).

We propose a model in which presence of trophic factors in the ECM, and/or their increased expression by astrocytes after brain damage might interact with specific receptors expressed by perivascular infiltrating mononuclear cells or macrophages inducing a partial suppression of the immune response (Fig. 6). This effect might be achieved by several mechanisms: (i) closing the BBB and diminishing the trafficking of activated inflammatory monocytes, via actions of NGF on brain perivascular mast cells or on expression of molecules responsible for lymphocyte adhesion and migration, (ii) inducing a switch of T cell phenotype from a Th1 phenotype to a Th2 phenotype, (iii) inducing the expression of immunosuppressive cytokines such as IL-10, and perhaps TGF- β , (iv) reducing activation of macrophages and microglial cells and downregulating expression of MHC class II molecules on these cells, (v) inducing the inactivation of toxic immune mediators by astrocytes, and (vi) modulating overall immune activity through sympathetic innervation. Several of these proposed mechanisms have been demonstrated, however comprehensive studies will be needed to understand the complex and dynamic nature of these processes. A fundamental point that arises from studies performed to date is that the effects of NGF must be seen as those of a pleiotropic cytokine in contrast to other specialized cytokines, and that overall effects will be the summary of NGF

signaling in the different systems expressing NGF receptors.

In addition to the immunosuppressive properties, trophic factors such as NGF most certainly contribute to the protection of injured neurons and oligodendrocytes, and promote survival and regeneration. This neuroprotective role may result from priming of anti-apoptotic or cell cycle progression pathways such as NF κ B or Ras (Lopez-Sanchez and Frade, 2002). Although the effects of trophic factors in rescuing damaged neurons and oligodendrocytes are clearly demonstrated, little is known of their role in protecting demyelinated or damaged axons. This effect could be very important in MS, considering recent neuropathological analyses that show early axonal damage in this disease (Trapp et al., 1999). Thus, trophic factor networks might have more pleiotropic roles in maintaining homeostasis of the adult CNS than the very specific functions which they support during development.

Therapeutics opportunities for NGF in inflammatory brain diseases

The findings that NGF induces immunosuppression that is CNS-specific during autoimmune demyelination, in addition to its neuroprotective properties for oligodendrocytes and neurons, make this protein an attractive candidate molecule for treatment of CNS inflammatory diseases. Neurotrophic factors have been successfully used to treat animal models of central and peripheral nervous system disease with the rational of protecting neurons, axons and myelin (Yuen and Mobley, 1996; Kernie and Parada, 2000). Sadly, attempts to translate such therapies to human

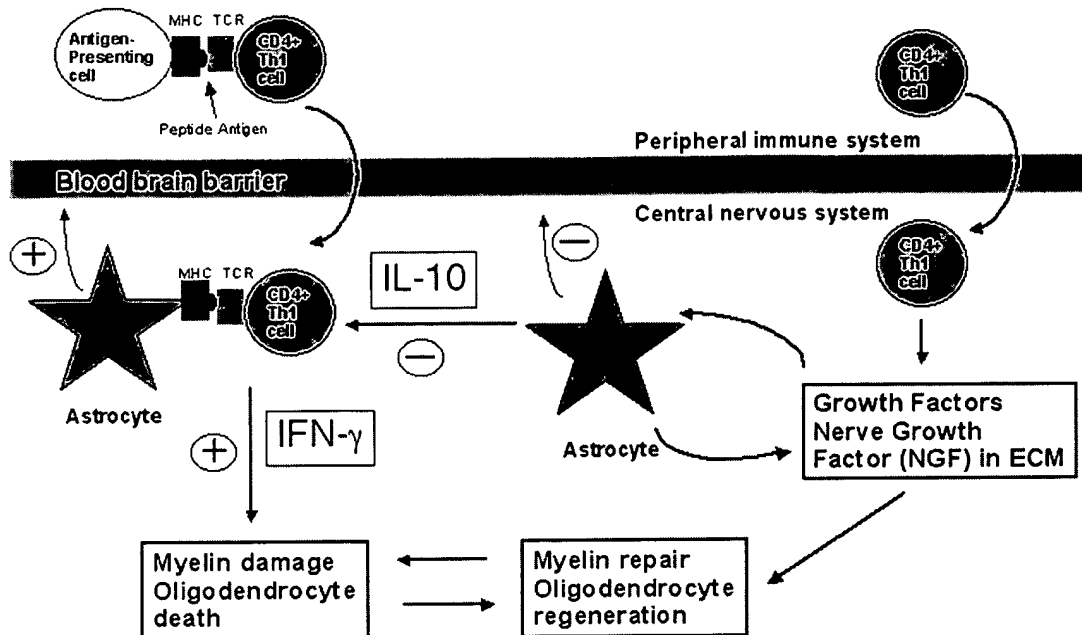


Fig. 6. Schematic model for the role of trophic factor networks in maintenance of CNS immune privilege. Right, simplified pathway of inflammation and myelin damage (demyelination) by myelin antigen-specific CD4⁺ Th1 cells that migrate into the CNS parenchyma and are reactivated by antigen presentation in context of MHC class II molecules expressed on astrocytes (represented in light gray). T cell, macrophage and glial cell activation further contributes to blood-brain barrier (BBB) permeability. Left, NGF and growth factors are secreted by both astrocytes and certain infiltrating T cells. In addition to their neuroprotective effects, these growth factors can stimulate astrocytes to produce IL-10, a suppressive cytokine that has the potential to downregulate inflammatory process and decrease BBB permeability (represented in dark gray).

diseases have failed to date, decreasing the current interest in these factors as therapeutics agents for neurological diseases (Apfel, 2002). Inherent weakness in most clinical trials are the use of systemic administration which results in failure to cross the BBB, and unacceptable side effects of treatment which are dose-limiting. A more comprehensive approach to disease pathogenesis that accounts for mechanisms of action of neurotrophins, and means of targeted delivery to specific organs might allow the development of promising NGF-based therapies for neurological diseases.

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Abbreviations

BBB	blood-brain barrier
BDNF	brain derived neurotrophic factor
CIS	clinically isolated syndrome
CNS	central nervous system
CNTF	ciliary neurotrophic factor
EAE	experimental allergic encephalomyelitis
ECM	extracellular matrix
GDNF	glial derived neurotrophic factor
GGF	glial growth factor
GMCSF	granulocyte monocyte colony stimulating factor
IL-	interleukin-
IFN-	interferon-
IGF	insulin-like growth factor

433	LIF	leukemia inhibitory factor
434	LPS	lipopolysaccharide
435	MHC	major histocompatibility complex
436	MS	multiple sclerosis
437	NGF	nerve growth factor
438	PBMC	peripheral blood mononuclear cells
439		
440	p75 ^{NTR} p75	neurotrophin receptor
441	RR-,SP-,PP-MS	relapsing remitting, secondary progressive, primary progressive multiple sclerosis
442		
443		
444	TGF	transforming growth factor
445	Th	T helper cell
446	TNF	tumor necrosis factor
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References

- 450
- 451 Aloe, L., Skaper, S.D., Leon, A. and Levi-Montalcini, R.
- 452 (1994) Nerve growth factor and autoimmune diseases.
- 453 Autoimmunity, 19: 141–150.
- 454 Antel, J.P. and Owens, T. (1999) Immune regulation and CNS
- 455 autoimmune disease. *J. Neuroimmunol.*, 100: 181–189.
- 456 Apfel, S.C. (2002) Is the therapeutic application of neuro-
- 457 trophic factors dead? *Ann. Neurol.*, 51: 8–11.
- 458 Arredondo, L.R., Deng, C., Ratts, R.B., Lovett-Racke, A.E.,
- 459 Holtzman, D.M. and Racke, M.K. (2001) Role of nerve
- 460 growth factor in experimental autoimmune encephalomyeli-
- 461 tis. *Eur. J. Immunol.*, 31: 625–633.
- 462 Awatsuji, H., Furukawa, Y., Hirota, M., Furukawa, S. and
- 463 Hayashi, K. (1995) Interferons suppress nerve growth factor
- 464 synthesis as a result of interference with cell growth in
- 465 astrocytes cultured from neonatal mouse brain.
- 466 *J. Neurochem.*, 64: 1476–1482.
- 467 Awatsuji, H., Furukawa, Y., Hirota, M., Murakami, Y., Nii, S.,
- 468 Furukawa, S. and Hayashi, K. (1993) Interleukin-4 and -5 as
- 469 modulators of nerve growth factor synthesis/secretion in
- 470 astrocytes. *J. Neurosci. Res.*, 34: 539–545.
- 471 Bettelli, E., Das, M., Howard, E., Weiner, H., Sobel, R. and
- 472 Kuchroo, V. (1998) IL-10 is critical in the regulation of
- 473 autoimmune encephalomyelitis as demonstrated by studies of
- 474 IL-10- and IL-4-deficient and transgenic mice. *J. Immunol.*,
- 475 161: 3299–3306.
- 476 Boutros, T., Croze, E. and Yong, V.W. (1997) Interferon-beta
- 477 is a potent promoter of nerve growth factor production by
- 478 astrocytes. *J. Neurochem.*, 69: 939–946.
- 479 Bradl, M. and Lington, C. (1996) Animal models of
- 480 demyelination. *Brain Pathol.*, 6: 303–311.
- Brodie, C. and Gelfand, E.W. (1992) Functional nerve growth factor receptors on human B lymphocytes. Interaction with IL-2. *J. Immunol.*, 148: 3492–3497.
- Brosnan, C.F. and Raine, C.S. (1996) Mechanisms of immune injury in multiple sclerosis. *Brain Pathol.*, 6: 243–257.
- Bullock, E. and Johnson, E., Jr. (1996) Nerve growth factor induces the expression of certain cytokine genes and bcl-2 in mast cells. Potential role in survival promotion. *J. Biol. Chem.*, 271.
- Butzkueven, H., Zhang, J., Soilu-Hanninen, M., Hochrein, H., Chionh, F., Shipham, K., Emery, B., Turnley, A., Petratos, S., Ernst, M., Bartlett, P. and Kilpatrick, T. (2002) LIF receptor signaling limits immune-mediated demyelination by enhancing oligodendrocyte survival. *Nat. Med.*, 8: 613–619.
- Calza, L., Giardino, L., Pozza, M., Bettelli, C., Micera, A. and Aloe, L. (1998) Proliferation and phenotype regulation in the subventricular zone during experimental allergic encephalomyelitis: in vivo evidence of a role for nerve growth factor. *Proc. Natl. Acad. Sci. USA*, 95: 3209–3214.
- Cannella, B., Gao, Y.L., Brosnan, C. and Raine, C.S. (1996) IL-10 fails to abrogate experimental autoimmune encephalomyelitis. *J. Neurosci. Res.*, 45: 735–746.
- Cannella, B., Hoban, C.J., Gao, Y.L., Garcia-Arenas, R., Lawson, D., Marchionni, M., Gwynne, D. and Raine, C.S. (1998) The neuregulin, glial growth factor 2, diminishes autoimmune demyelination and enhances remyelination in a chronic relapsing model for multiple sclerosis. *Proc. Natl. Acad. Sci. USA*, 95: 10100–10105.
- Caroleo, M.C., Costa, N., Bracci-Laudiero, L. and Aloe, L. (2001) Human monocyte/macrophages activate by exposure to LPS overexpress NGF and NGF receptors. *J. Neuroimmunol.*, 113: 193–201.
- Carr, D. and Campbell, I. (1999) Transgenic expression of interleukin-6 in the central nervous system confers protection against acute herpes simplex virus type-1 infection. *J. Neurovirol.*, 5: 449–457.
- Casaccia-Bonnel, P., Carter, B.D., Dobrowsky, R.T. and Chao, M.V. (1996) Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. *Nature*, 383: 716–719.
- Chen, Y., Kuchroo, V.K., Inobe, J.-I., Hafler, D.A. and Weiner, H.L. (1994) Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science*, 265: 1237–1240.
- Cua, D., Groux, H., Hinton, D., Stohlman, S. and Coffman, R. (1999) Transgenic interleukin 10 prevents induction of experimental autoimmune encephalomyelitis. *J. Exp. Med.*, 189: 1005–1010.
- De Simone, R., Micera, A., Tirassa, P. and Aloe, L. (1996) mRNA for NGF and p75 in the central nervous system of rats affected by experimental allergic encephalomyelitis. *Neuropathol. Appl. Neurobiol.*, 22: 54–59.

- de Webster, H. (1997) Growth factors and myelin regeneration in multiple sclerosis. *Mult. Scler.*, 3: 113–120.
- Dowling, P., Ming, X., Raval, S., Husar, W., Casaccia-Bonnel, P., Chao, M., Cook, S. and Blumberg, B. (1999) Up-regulated p75NTR neurotrophin receptor on glial cells in MS plaques. *Neurology*, 53: 1676–1682.
- Ehrhard, P.B., Erb, P., Graumann, U. and Otten, U. (1993) Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. *Proc. Natl. Acad. Sci. USA*, 90: 10984–10988.
- Flugel, A., Matsumuro, K., Neumann, H., Klinkert, W.E., Birnbacher, R., Lassmann, H., Otten, U. and Wekerle, H. (2001) Anti-inflammatory activity of nerve growth factor in experimental autoimmune encephalomyelitis: inhibition of monocyte transendothelial migration. *Eur. J. Immunol.*, 31: 11–22.
- Frade, J.M., Rodriguez-Tebar, A. and Barde, Y.A. (1996) Induction of cell death by endogenous nerve growth factor through its p75 receptor. *Nature*, 383: 166–168.
- Frei, K., Lins, H., Schwerdel, C. and Fontana, A. (1994) Antigen presentation in the central nervous system. The inhibitory effect of IL-10 on MHC class II expression and production of cytokines depends on the inducing signals and the type of cell analyzed. *J. Immunol.*, 152: 2720–2728.
- Friedman, W.J., Thakur, S., Seidman, L. and Rabson, A.B. (1996) Regulation of nerve growth factor mRNA by interleukin-1 in rat hippocampal astrocytes is mediated by NF κ B. *J. Biol. Chem.*, 271: 31115–31120.
- Gadient, R.A., Cron, K.C. and Otten, U. (1990) Interleukin-1 beta and tumor necrosis factor- α synergistically stimulate nerve growth factor (NGF) release from cultured rat astrocytes. *Neurosci. Lett.*, 117: 335–340.
- Genain, C. and Hauser, S. (2001) Experimental allergic encephalomyelitis in the common marmoset *C. jacchus*. *Immunol. Rev.*, 183: 159–172.
- Genain, C.P., Abel, K., Belmar, N., Villinger, F., Rosenberg, D.P., Linington, C., Raine, C.S. and Hauser, S.L. (1996) Late complications of immune deviation therapy in a nonhuman primate. *Science*, 274: 2054–2057.
- Kernie, S.G. and Parada, L.F. (2000) The molecular basis for understanding neurotrophins and their relevance to neurologic disease. *Arch. Neurol.*, 57: 654–657.
- Kerschensteiner, M., Gallmeier, E., Behrens, L., Leal, V., Misgeld, T., Klinkert, W., Kolbeck, R., Hoppe, E., Oropeza-Wekerle, R., Bartke, I., Stadelmann, C., Lassmann, H., Wekerle, H., Hohlfeld, R. (1999) Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a neuroprotective role of inflammation. *J. Exp. Med.*, 189: 865–870.
- Kramer, R., Zhang, Y., Gehrmann, J., Gold, R., Thoenen, H. and Wekerle, H. (1995) Gene transfer through the blood-nerve barrier: NGF-engineered neuritogenic T lymphocytes attenuate experimental autoimmune neuritis. *Nat. Med.*, 1: 1162–1166.
- Laudiero, L.B., Aloe, L., Levi-Montalcini, R., Buttinelli, C., Schilter, D., Gillesen, S. and Otten, U. (1992) Multiple sclerosis patients express increased levels of beta-nerve growth factor in cerebrospinal fluid. *Neurosci. Lett.*, 147: 9–12.
- Levi-Montalcini, R., Skaper, S., Dal Toso, R., Petrelli, L. and Leon, A. (1996) Nerve growth factor: from neurotrophin to neurokine. *Trends Neurosci.*, 19: 514–520.
- Linker, R.A., Maurer, M., Gaupp, S., Martini, R., Holtmann, B., Giess, R., Rieckmann, P., Lassmann, H., Toyka, K.V., Sendtner, M., Gold, R. (2002) CNTF is a major protective factor in demyelinating CNS disease: a neurotrophic cytokine as modulator in neuroinflammation. *Nat. Med.*, 8: 620–624.
- Lopez-Sanchez, N. and Frade, J.M. (2002) Control of the cell cycle by neurotrophins: lessons from the p75 neurotrophin receptor. *Histol. Histopathol.*, 17: 1227–1237.
- Lovett-Racke, A.E., Bittner, P., Cross, A.H., Carlino, J.A. and Racke, M.K. (1998) Regulation of experimental autoimmune encephalomyelitis with insulin-like growth factor (IGF-1) and IGF-1/IGF-binding protein-3 complex (IGF-1/IGFBP3). *J. Clin. Invest.*, 101: 1797–1804.
- Melamed, I., Kelleher, C.A., Franklin, R.A., Brodie, C., Hempstead, B., Kaplan, D. and Gelfand, E.W. (1996) Nerve growth factor signal transduction in human B lymphocytes is mediated by gp140trk. *Eur. J. Immunol.*, 26: 1985–1992.
- Micera, A., Properzi, F., Triaca, V. and Aloe, L. (2000) Nerve growth factor antibody exacerbates neuropathological signs of experimental allergic encephalomyelitis in adult lewis rats. *J. Neuroimmunol.*, 104: 116–123.
- Micera, A., Vigneti, E. and Aloe, L. (1998) Changes of NGF presence in non neuronal cells in response to experimental allergic encephalomyelitis in Lewis rats. *Exp. Neurol.*, 154: 41–46.
- Moalem, G., Gdalyahu, A., Shani, Y., Otten, U., Lazarovici, P., Cohen, I.R. and Schwartz, M. (2000) Production of neurotrophins by activated T cells: implications for neuroprotective autoimmunity. *J. Autoimmun.*, 15: 331–345.
- Muhallab, S., Lundberg, C., Gielen, A.W., Lidman, O., Svenningsson, A., Piehl, F. and Olsson, T. (2002) Differential expression of neurotrophic factors and inflammatory cytokines by myelin basic protein-specific and other recruited T cells infiltrating the central nervous system during experimental autoimmune encephalomyelitis. *Scand. J. Immunol.*, 55: 264–273.
- Neumann, H., Misgeld, T., Matsumuro, K. and Wekerle, H. (1998) Neurotrophins inhibit major histocompatibility class II inducibility of microglia: involvement of the p75 neurotrophin receptor. *Proc. Natl. Acad. Sci. USA*, 95: 5779–5784.
- O'Connell, J., Houston, A., Bennett, M.W., O'Sullivan, G.C. and Shanahan, F. (2001) Immune privilege or inflammation? Insights into the Fas ligand enigma. *Nat. Med.*, 7: 271–274.

- Oderfeld-Nowak, B., Zaremba, M., Micera, A. and Aloe, L. (2001) The upregulation of nerve growth factor receptors in reactive astrocytes of rat spinal cord during experimental autoimmune encephalomyelitis. *Neurosci. Lett.*, 308: 165–168.
- Racke, M.K., Bonomo, A., Scott, D.E., Cannella, B., Levine, A., Raine, C.S., Shevach, E.M. and Rocken, M. (1994) Cytokine-induced immune deviation as a therapy for inflammatory autoimmune disease. *J. Exp. Med.*, 180: 1961–1966.
- Racke, M.K., Dhib-Jalbut, S., Cannella, B., Albert, P.S., Raine, C.S. and McFarlin, D.E. (1991) Prevention and treatment of chronic relapsing experimental allergic encephalomyelitis by transforming growth factor-beta 1. *J. Immunol.*, 146: 3012–3017.
- Ransohoff, R. and Trebst, C. (2000) Surprising pleiotropy of nerve growth factor in the treatment of experimental autoimmune encephalomyelitis. *J. Exp. Med.*, 191: 1625–1629.
- Rodriguez, M., Pavelko, K., McKinney, C. and Leibowitz, J. (1994) Recombinant human IL-6 suppresses demyelination in a viral model of multiple sclerosis. *J. Immunol.*, 153: 3811–3821.
- Santambrogio, L., Benedetti, M., Chao, M.V., Muzaffar, R., Kulig, K., Gabellini, N. and Hochwald, G. (1994) Nerve growth factor production by lymphocytes. *J. Immunol.*, 153: 4488–4495.
- Schull, M., Ormsby, I. and Kier, A. (1992) Targeted disruption of the mouse transforming growth factor b gene results in multifocal inflammatory disease. *Nature*, 359: 693–699.
- Stadelmann, C., Kerschensteiner, M., Misgeld, T., Bruck, W., Hohlfeld, R. and Lassmann, H. (2002) BDNF and gp145trkB in multiple sclerosis brain lesions: neuroprotective interactions between immune and neuronal cells? *Brain*, 125: 75–85.
- Steinman, L. (2001) Multiple sclerosis: a two-stage disease. *Nat. Immunol.*, 2: 762–764.
- Streilein, J.W. (1995) Unraveling immune privilege. *Science*, 270: 1158–1159.
- Streilein, J.W., Ksander, B.R. and Taylor, A.W. (1997) Immune deviation in relation to ocular immune privilege. *J. Immunol.*, 158: 3557–3560.
- Torcia, M., Bracci-Laudiero, L., Lucibello, M., Nencioni, L., Labardi, D., Rubartelli, A., Cozzolino, F., Aloe, L. and Garaci, E. (1996) Nerve growth factor is an autocrine survival factor for memory B lymphocytes. *Cell*, 85: 345–356.
- Trapp, B., Bo, L., Mork, S. and Chang, A. (1999) Pathogenesis of tissue injury in MS lesions. *J. Neuroimmunol.*, 98: 49–56.
- Valdo, P., Stegagno, C., Mazzucco, S., Zuliani, E., Zanusso, G., Moretto, G., Raine, C.S. and Bonetti, B. (2002) Enhanced expression of NGF receptors in multiple sclerosis lesions. *J. Neuropathol. Exp. Neurol.*, 61: 91–98.
- Villoslada, P., Hauser, S., Bartke, I., Unger, J., Heald, N., Rosenberg, D., Cheung, S., Mobley, W., Fisher, S. and Genain, C. (2000) Human nerve growth factor protects common marmosets against autoimmune encephalomyelitis by switching the balance of T helper type 1 and 2 cytokines within the central nervous system. *J. Exp. Med.*, 191: 1799–1806.
- Willenborg, D., Fordhan, S., Cowden, W. and Ramshaw, I. (1995) Cytokines and murine autoimmune encephalomyelitis. Inhibition or enhancement of disease with antibodies to select cytokines, or by delivery of exogenous cytokines using a recombinant vaccinia virus system. *Scand. J. Immunol.*, 41: 31–41.
- Yuen, E. and Mobley, W. (1996) Therapeutic potential of neurotrophic factors for neurological disorders. *Ann. Neurol.*, 40: 346–354.

Oligodendrocyte precursor cells in the demyelinated multiple sclerosis spinal cord

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Summary

Lesions appearing in the CNS of patients in the chronic phase of the inflammatory, demyelinating disease multiple sclerosis often fail to repair, resulting in neurological dysfunction. This failure of remyelination appears, in many cases, to be due not to the destruction of the local oligodendrocyte precursor population, a source for new myelin-forming cells, but to the failure of the precursor cells to proliferate and differentiate, at least in brain lesions. The spinal cord is also a prominent site for lesions in multiple sclerosis, but nothing is known about the fate of the oligodendrocyte precursor population in this area. The present study has therefore analysed spinal cord samples with demyelination from 16 subjects with longstanding multiple sclerosis for the presence of oligodendrocyte precursor cells. Immunolabellings of 10 µm thick sections with the O4/anti-galactocerebroside (GalC) antibody combination, to visualize O4-positive, GalC-negative oligodendrocyte precursor cells, revealed that such cells were prevalent in many spinal cord lesions, with densities of up to 35 cells/mm². Six of the spinal cord lesions contained ≤ 3 O4-positive, GalC-negative cells/mm², but such cells were widespread in brain lesions from these multiple sclerosis cases that were available for study (8–26 cells/mm²). The density of the O4-positive, GalC-negative oligodendrocyte precursor cells in all spinal cord and

brain lesions studied thus far ($n = 41$) decreased significantly with declining numbers of debris-laden macrophages. In addition, lesions lacking macrophages tended to be derived from the older patients and there was a negative correlation between the density of the oligodendrocyte precursor cells and clinical age of the multiple sclerosis subject at death, and disease duration. The analysis further revealed that lesions from subjects with primary progressive and secondary progressive multiple sclerosis contained, on average, similar numbers of oligodendrocyte precursor cells/mm² and that immature oligodendrocytes were only present in significant numbers in lesions with high precursor densities. Taken together, the present data suggest that there is a gradual reduction in the size of the O4-positive, GalC-negative oligodendrocyte precursor population with increasing age of the lesion, that the generation of new oligodendrocytes becomes increasingly more impaired and that lesions are not repopulated to a significant extent by migratory oligodendrocyte precursor cells present in the adjacent unaffected tissue. Hence, strategies intended to promote endogenous remyelination in multiple sclerosis patients should focus on both enhancing the long-term survival of oligodendrocyte precursor cells and on stimulating these cells to proliferate and differentiate into remyelinating oligodendrocytes.

Keywords: demyelination; multiple sclerosis; O4; oligodendrocyte precursor cell; remyelination

Abbreviations: GalC = galactocerebroside; GFAP = glial fibrillary acidic protein; HLA = human leucocyte antigen; MBP = myelin basic protein; MOG = myelin oligodendrocyte glycoprotein; PDGF = platelet-derived growth factor; PP = primary progressive; RR = relapsing–remitting; SP = secondary progressive

Introduction

The generation of new myelin-forming oligodendrocytes in the inflammatory, demyelinating disease multiple sclerosis fails as disease progresses, resulting in the formation of confluent areas of persistent demyelination, impairment of impulse conduction along the denuded axons and neuro-

logical symptoms (Smith, 1996; Lassmann *et al.*, 1997; Prineas and McDonald, 1997; Lucchinetti *et al.*, 2000; Wingerchuk *et al.*, 2001). This situation is very different from that observed in experimental models of demyelinating disease where the generation of new oligodendrocytes, re-

Table 1 Details of multiple sclerosis subjects

MS case	NBB number	Age (years)	Gender	Duration of symptoms (years)	MS type	Maximum autolysis time (h:min)
1	96-039	57	F	19	PP	5:45
2	96-040	35	F	11	SP	5:45
3	97-160	40	F	10	SP	7:00
4	97-168	54	F	37	PP/SP	7:00
5	98-009	70	F	32	SP	6:30
6	98-066	75	F	27	PP/SP	4:50
7	98-087	55	F	19	SP	7:35
8	99-025	64	F	35	SP	7:45
9	99-054	58	F	20	SP	8:10
10	99-056	72	M	46	PP	8:30
11	99-066	69	M	27	PP/SP	16:45
12	99-073	71	F	24	PP/SP	8:20
13	99-086	71	F	24	RR	10:25
14	00-024	52	F	22	PP/SP	8:25
15	00-120	69	F	21	PP/SP	13:20
16	01-018	48	F	9	SP	8:10
17*	94-042	32	M	8	SP/PP	9:35
18	95-095	56	M	12	PP	5:25
19	96-025	34	F	10	SP	6:50
20	96-026	69	F	19	SP	9:15
21	96-074	40	F	14	SP	7:00
22	96-076	81	F	49	PP/SP	4:15
23	96-121	73	M	22	SP	4:45
24	97-070	82	F	25	PP	4:30
25	97-077	50	M	17	SP/PP	5:40
26	97-123	46	M	23	SP	3:45

*Brain lesions from multiple sclerosis cases 17–26 were analysed in a previous study (Wolswijk, 1998b); oligodendrocyte precursor and macrophage densities in these brain lesions are included in the data presented in Fig. 3.

ensheathment of denuded axons, restoration of impulse conduction and functional recovery frequently are complete (Ludwin, 1981; Jeffery and Blakemore, 1997; Wolswijk, 1998a; Franklin, 1999). The remyelinating cells in such models are generated by a population of immature cells capable of proliferation, migration and differentiation along the oligodendrocyte pathway (Ludwin, 1979; Arenella and Herndon, 1984; Godfraind *et al.*, 1989; Rodriguez, 1991; Carrol and Jennings, 1994; Gensert and Goldman, 1997; Franklin *et al.*, 1997; Keirstead *et al.*, 1998; Redwine and Armstrong, 1998; Di Bello *et al.*, 1999). The expansion of the oligodendrocyte precursor population and the production of new myelin-forming cells in response to demyelination is controlled by a number of growth factors, and their mRNAs are upregulated in distinct patterns during the remyelination process depending on whether they stimulate precursor proliferation and migration or promote oligodendrocytic differentiation (Wolswijk *et al.*, 1991; Komoly *et al.*, 1992; Tourbah *et al.*, 1992; Wolswijk and Noble, 1992; Yao *et al.*, 1995; Engel and Wolswijk, 1996; Shi *et al.*, 1998; Hinks and Franklin, 1999; Messersmith *et al.*, 2000). The success of repair of experimentally induced lesions is thought to be influenced by ageing, sex, the size of the lesion and the extent to which the oligodendrocyte precursor population is affected by the disease process (Gilson and Blakemore, 1993; Franklin *et al.*, 1997; Keirstead *et al.*, 1998; Shields *et al.*, 1999).

The limited success of myelin repair during the chronic phase of multiple sclerosis appears, in many cases, not to be the result of the concomitant destruction of both oligodendrocytes and their precursor cells. This notion has come from recent histopathological studies demonstrating that brain lesions from subjects with longstanding multiple sclerosis often contain substantial numbers of oligodendrocyte precursor cells, identified using the O4 antibody (Wolswijk, 1998b), antibodies to the platelet-derived growth factor (PDGF)- α receptor (Scolding *et al.*, 1998; Wolswijk, 1998b; Maeda *et al.*, 2001) and antibodies to the NG2 chondroitin sulphate proteoglycan (Chang *et al.*, 2000) (reviewed in Wolswijk, 1998a; Dawson *et al.*, 2000; Levine *et al.*, 2001). Although many oligodendrocyte precursor cells apparently survive the demyelination process in chronic stage multiple sclerosis, they appear to be in a relatively quiescent state (Wolswijk, 1998b, 2000). This finding raises the possibility that remyelination in chronic multiple sclerosis is scanty or absent because of the failure of the local oligodendrocyte precursor population to expand and generate new myelin-forming oligodendrocytes. Since lesion repair is more successful during the early course of multiple sclerosis (Prineas *et al.*, 1989; Raine and Wu, 1993; Prineas and McDonald, 1997; Lucchinetti *et al.*, 2000), it suggests that the proliferation and differentiation of oligodendrocyte precursor cells become gradually more impaired with progression of the

disease. Thus, the lesion environment changes from one conducive to remyelination to one hampering endogenous repair processes, because of either the absence of growth factors implicated in remyelination, the presence of inhibitory molecules or the presence of the scar tissue formed by astrocytes (Wolswijk, 1998a). Another intriguing possibility that has emerged from a recent study in the rat is that the therapeutic administration of glucocorticoids may impair the proliferative capacity of the oligodendrocyte precursor population (Alonso, 2000).

Demyelinated lesions also develop in the spinal cord of many multiple sclerosis patients and, because of their location, they can have devastating consequences in terms of disability (Smith, 1996; Prineas and McDonald, 1997). To gain further insights into the failure of lesion repair in multiple sclerosis, the present study has analysed demyelinated spinal cord samples obtained at autopsy from 16 subjects with longstanding multiple sclerosis for the presence of oligodendrocyte precursor cells, using indirect immunofluorescence techniques and confocal laser scanning microscopy.

Material and methods

Treatment of post-mortem spinal cord tissue

Spinal cord and brain tissue was obtained from The Netherlands Brain Bank (NBB; co-ordinator, R. Ravid); the NBB received permission for performing autopsies, for the use of tissue and for the access to medical records for research purposes from the Ethical Committee of the Medical Faculty of the Free University, Amsterdam, The Netherlands. Within 4 h 50 min–16 h 45 min after death (mean 8 h 25 min \pm 3 h 00 min), spinal cord samples (5–10 mm in length) and brain lesions from subjects with longstanding multiple sclerosis (Table 1) were placed in a solution of 4% paraformaldehyde in PBS (phosphate-buffered saline, pH 7.4), stored for 1–7 days at 4°C, and incubated in a solution of 30% sucrose in PBS for 1–3 days at 4°C under constant rotation. The tissue was then placed into a boat prepared from aluminium foil and filled with Tissue-Tek optimum cutting temperature embedding compound (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands), frozen on dry ice and stored at –80°C (Wolswijk, 1998b, 2000). Separate blocks of brain and spinal cord tissue were processed for neuropathological examination by Dr W. Kamphorst, Department of Pathology, Academic Hospital of the Free University, Amsterdam, The Netherlands.

Immunohistochemistry

Sections of 10 μ m were cut from each tissue block using a Reichert-Jung 2800 cryostat (cutting temperature –20 to –25°C), mounted onto SuperFrost*Plus microscope slides (Menzel-Gläser, Braunschweig, Germany) and immunolabelled, either directly or after storage at –20°C, using either

indirect immunofluorescence or immunoperoxidase techniques, as described before (Wolswijk, 1998b, 2000). Sections were incubated for 1–7 days at 4°C in the primary antibody solutions, rinsed several times in TBS (Tris-buffered saline, pH 7.6) and then incubated for 2 h at room temperature or overnight at 4°C with the fluorochrome [FITC (fluorescein isothiocyanate), TRITC (tetramethylrhodamine isothiocyanate), Cy3 or Cy5]-conjugated or biotinylated anti-rabbit or mouse IgG (H+L), or anti-mouse Ig subclass-specific antibodies (purchased from either Southern Biotechnology Associates, Inc., Birmingham, Ala., USA or Jackson ImmunoResearch, West Grove, Pa., USA). The binding of the biotinylated antibodies was visualized by incubating sections in the presence of the Vectastain ABC kit reagents A and B (Vector Laboratories, Inc., Burlingame, Calif., USA) followed by substrate {a filtered solution of 0.42 mg/ml 3-amino-9-ethylcarbazole [dissolved in dimethyl formamide (Merck, Darmstadt, Germany)] and 0.01% H₂O₂ (Merck) in 0.05 M sodium acetate (Sigma Chemical Company, St Louis, Miss., USA) buffer, pH 5.0} or by incubating sections in the presence of fluorochrome-coupled streptavidin (Vector). The primary antibodies used in the present study were: (i) the mouse O4 monoclonal antibody (Sommer and Schachner, 1981); (ii) a mouse IgG₃ anti-galactocerebroside (GalC) monoclonal antibody (the Ranscht antibody; Ranscht *et al.*, 1982); (iii) a rabbit antiserum to myelin basic protein (MBP; a gift from Dr H. van Noort, TNO, Leiden, The Netherlands); (iv) a mouse IgG₁ anti-myelin oligodendrocyte glycoprotein (MOG) antibody [the Y10 antibody (Piddlesden *et al.*, 1993), a gift from Dr S. Piddlesden]; (v) a rabbit antiserum to the NG2 chondroitin sulphate proteoglycan (a gift from Dr J. Levine, State University of New York, Stony Brook, NY, USA); (vi) an IgG_{2a} mouse monoclonal antibody to chondroitin sulphate proteoglycan (the 9.2.27 antibody; PharMingen, San Diego, Calif., USA); (vii) a rabbit antiserum to the PDGF- α receptor [the R7 rabbit polyclonal antibody raised against a synthetic peptide from the C-terminal end, which was also used in the study of Scolding *et al.*, 1998]; a gift of Dr C.-H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden]; (viii) a mouse IgG₁ anti-neurofilament monoclonal antibody [the RT97 antibody (Wood and Anderton, 1981); Boehringer Mannheim, Germany]; and (ix) a mouse IgG₁ anti-human leucocyte antigen (HLA)-DP, DQ, DR antigen (major histocompatibility complex class II) monoclonal antibody (Dako A/S, Glostrup, Denmark). Antibodies were diluted in TBS containing 0.25% Triton X-100 (Sigma) and/or 5% heat-inactivated calf serum (Sigma). Nuclei were visualized by incubating sections in 1 mg/ml Hoechst 33258 (Sigma) (for conventional fluorescence microscopical analysis), in 1 mM TO-PRO-3 iodide (Molecular Probes, Eugene, Oreg., USA) (for confocal laser scanning microscopical analysis) or in a haematoxylin solution (for bright-field microscopical analysis). At the end of the staining procedure, a drop of glycerol containing 22 mM 1,4-diazobicyclo [2,2,2] octane (Sigma) was placed on the section (to reduce fading of the

fluorochromes), followed by a glass coverslip. The excess glycerol was removed and the coverslip was then sealed using clear nail varnish. Sections were viewed on a Zeiss Axiophot

microscope equipped with phase-contrast, bright-field and dark-field optics, epi-UV illumination and selective filters optimized for distinguishing between FITC and TRITC/Cy3

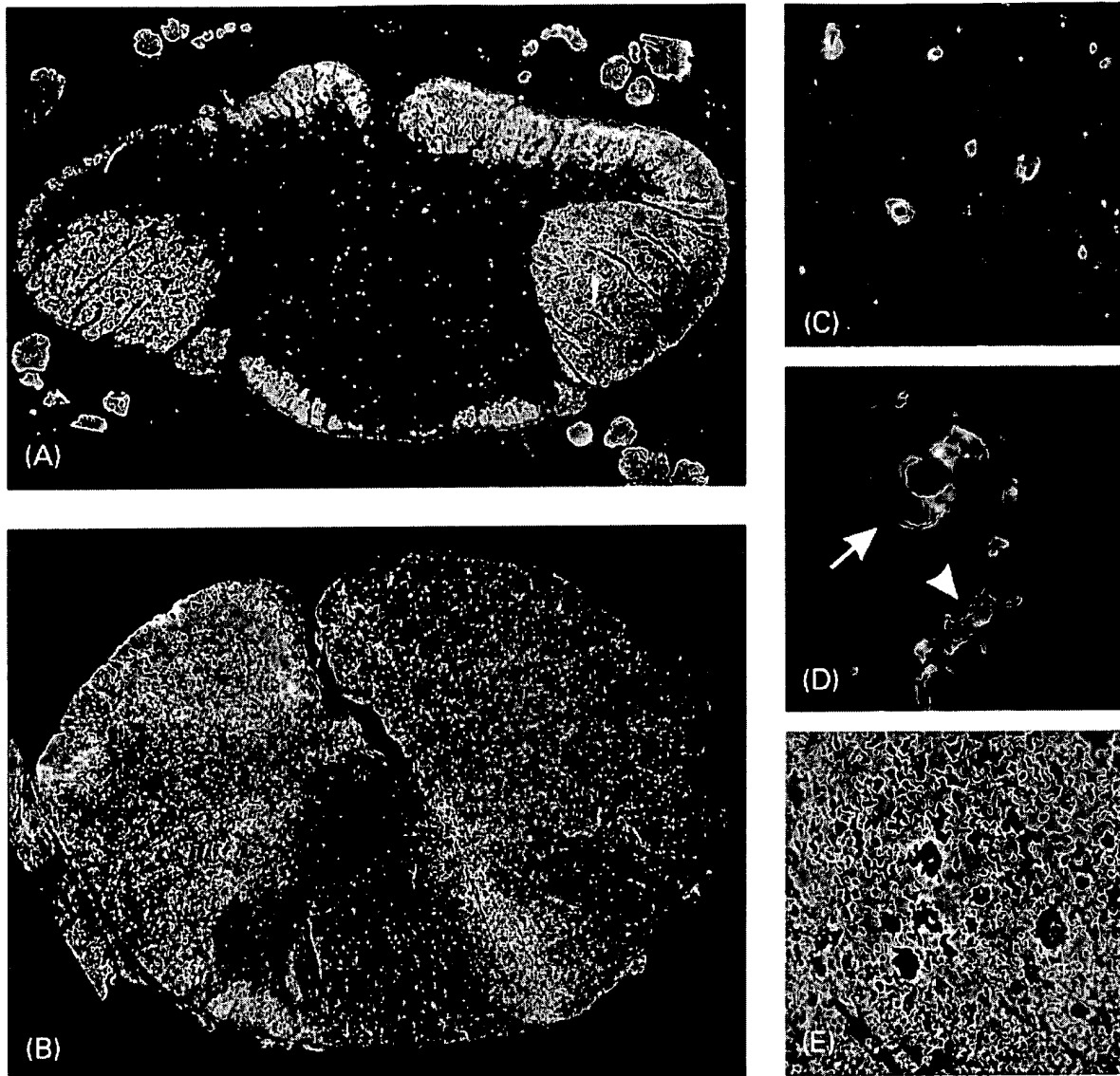


Fig. 1 Details of multiple sclerosis spinal cord samples with demyelination. (A) Low power view of a cross-section through a multiple sclerosis spinal cord (Case 5) immunolabelled with antibodies to myelin basic protein (MBP; green, FITC) and neurofilament (red, TRITC), an intermediate filament found in axons. Note that the central portion of this spinal cord section, including the grey matter, contains virtually only demyelinated axons, (see also Table 2). Very few debris-laden macrophages were present in this spinal cord sample (Table 3), suggesting that the demyelinating activity had occurred some time ago. Diameter of the spinal cord, 7 mm. (B) Low power view of a section cut from the spinal cord sample from multiple sclerosis case 14 that was immunolabelled with antibodies to HLA-DP, DQ, DR antigens (green, FITC), which label activated cells of the microglial/macrophage lineage, and antibodies to MBP (red, Cy3). The activated microglia/macrophages were found mostly at the borders of the lesional areas. Note that the grey matter is partially affected by the demyelination process (Table 2). Diameter of the spinal cord, 9 mm. (C) Detail of an area of a multiple sclerosis spinal cord (Case 3) with very few myelinated axons. Axons were visualized with antibodies to neurofilament (red, TRITC), while the myelin rings were visualized with antibodies to MBP (green, FITC). Image size, 200 μm \times 200 μm . (D) HLA-DP, DQ, DR-positive microglia/macrophages (arrow; green, FITC) were sometimes observed within layers of MBP-positive myelin segments (red, Cy3); nuclei were visualized with the nuclear dye TO-PRO-3 iodide (blue). Immunoreactivity for HLA-DP, DQ, DR is also visible in another myelin ring (arrowhead). Multiple sclerosis case 9. Image size, 35 μm \times 35 μm . (E) Most demyelinated areas of the spinal cord samples were packed with GFAP-positive filaments (green, FITC), an intermediate filament found in astrocytes. The small numbers of myelin sheaths in the lesion area shown were labelled with antibodies to GalC (red, TRITC). Multiple sclerosis case 3. Image size, 200 μm \times 200 μm .

Table 2 Details of spinal cord samples with demyelination

MS case	% Spinal cord section		Extent of grey matter involvement*	% Spinal cord section with phase-bright macrophages	Highest density of phase-bright macrophages (cells/mm ²)
	Completely demyelinated	Affected			
1	>95	100	+++	<5	200–250
2	70–80	>95	+++	40–50	900–950
3	50–60	>95	++	<5	250–300
4	30–40	>95	+++	<1	ND
5	60–70	>95	+++	5–10	250–300
6	<5	>5	+	<5	650–700
7 ^a	50–60	>90	+++	20–30	650–700
7b	50–60	>95	+++	10–20	400–450
8	10–20	>20	+	<1	ND
9	50–60	>70	++	10–20	650–700
10	80–90	>90	++	<1	ND
11	10–20	>20	+	<1	ND
12	10–20	>20	++	<1	ND
13	10–20	>30	++	<5	200–250
14	30–40	>40	++	10–20	400–450
15	>95	100	+++	<1	ND
16	10–20	>60	+++	<5	250–300

*The grey matter area of the spinal cord samples studied either had some demyelination (+) or were (almost) completely demyelinated (+++). ^aTwo different spinal cord samples (a and b) from multiple sclerosis subject 7 were analysed.

and Hoechst emission, or on a Zeiss 410 inverted confocal laser scanning microscope with three different lasers emitting at 488, 543 and 633 nm to excite FITC, TRITC/Cy3 and TO-PRO-3 iodide/Cy5, respectively, and with bright-field optics.

Cell counts and data analysis

The density of the populations of O4-positive, GalC-negative oligodendrocyte precursor cells, process-bearing GalC-positive oligodendrocytes and phase-bright macrophages in completely demyelinated areas of the spinal cord sections was calculated from the number of cells present in microscope fields with a size of 1/16 (0.0625) mm² [≥ 30 fields/section ($n = 3$ sections)]. Depending on the size of the lesion area, either every adjacent microscope field or up to every fifth field was analysed. Microcal Origin 5.0 software was used to plot and analyse the data.

Results

Characteristics spinal cord tissue with demyelination

Seventeen spinal cord samples (5–10 mm in length) from 16 subjects with chronic multiple sclerosis (Table 1) were analysed for the presence of oligodendrocyte precursor cells. The collection included samples from subjects with relapsing–remitting (RR) multiple sclerosis (1 case), primary progressive (PP) multiple sclerosis (two cases) and secondary progressive (SP) multiple sclerosis (seven cases); the remaining six subjects died during the progressive phase of the

disease, but it was not clear from their medical records whether they had suffered from the PP or SP form of multiple sclerosis. The 17 blocks had areas of complete demyelination ranging from <5% to >95% of a complete spinal cord cross-section, including the grey matter region (Fig. 1 and Table 2). These areas contained numerous axons, identified using antibodies to neurofilament, but it is likely that axon loss had occurred in these regions, either because of injury occurring in the lesion area itself or because of the presence of other lesions along the length of the spinal cord (secondary Wallerian degeneration) (Prineas and McDonald, 1997). Areas with reduced numbers of myelinated axons with or without demyelinated axons were observed in most samples and, in seven cases, >95% of the cross-section was affected by the disease process (Table 2). None of the spinal cord samples contained significant areas lacking both myelin and axons, i.e. areas larger than the size of a microscope field of 0.0625 mm². Mature oligodendrocytes lacking myelin-forming processes were either absent or present in only small numbers in the affected regions. Remyelinated axons, i.e. axons surrounded by weakly MOG-positive, thin myelin sheaths, were rarely observed. The lesion areas were packed with glial fibrillary acidic protein (GFAP)-positive filaments (Fig. 1), an intermediate filament found in astrocytes.

Numerous macrophages filled with myelin degradation products were present in 11 of the 17 blocks and they occupied up to 50% of the spinal cord cross-sections, with densities of up to 900–950 cells/mm² section (10 μ m thick) (Table 2). They were distributed either throughout the lesion area, concentrated at the lesion edges (see Fig. 1) or present in

small clusters in areas with still many myelinated axons. Immunolabellings involving antibodies to HLA-DP, DQ, DR [major histocompatibility complex class II] antigens showed that most samples contained activated cells of the microglia/macrophage lineage (Fig. 1). Confocal laser scanning microscopic analysis revealed many examples in which the activated microglia/macrophages appeared to have engulfed individual myelin rings, or contained either MBP-positive myelin fragments or diffuse MBP immunoreactivity. Moreover, HLA-DP, DQ, DR-positive cells were sometimes observed within layers of the myelin sheaths (Fig. 1). The myelin-free areas of the sections contained lower numbers of phase-bright macrophages (Table 3) and these cells tended to lack immunoreactivity for MBP.

Oligodendrocyte precursor cells in demyelinated spinal cord lesions

The multiple sclerosis spinal cord sections were immunolabelled with three different antibody markers for oligodendrocyte precursor cells, i.e. the O4 monoclonal antibody (Sommer and Schachner, 1981) [in combination with antibodies to GalC to distinguish between O4-positive, GalC-negative oligodendrocyte precursor cells and O4-positive, GalC-positive oligodendrocytes (Wolswijk, 1998b)] and antibodies to the PDGF- α receptor and NG2 chondroitin sulphate proteoglycan. Consistent and reliable labelling in all spinal cord samples (and spinal nerves) of oligodendrocyte lineage cells and/or of myelin was observed only with the O4 antibody (Fig. 2), with no obvious deleterious effects of autolysis time of the tissue and length of fixation (see also Back *et al.*, 2001). With two different antibodies to NG2 [a rabbit antiserum and the 9.2.27 monoclonal antibody used by Chang *et al.* (2000)], only consistent labelling was found of blood vessels and of cells in the spinal nerves (Fig. 2), which are probably non-myelinating Schwann cells (Schneider *et al.*, 2001). Only very occasionally, a cell with an oligodendrocyte precursor-like morphology expressed NG2 in the spinal cord sections. NG2-labelling in the spinal cord sections was, however, only seen following long incubations with the primary antibody and using an amplification step (Chang *et al.*, 2000). Using this improved immunolabelling protocol, NG2-positive, oligodendrocyte precursor-like cells were now observed in brain lesions derived from multiple sclerosis cases 13 and 14 (Fig. 2), in contrast to that reported previously (Wolswijk, 1998b). These results suggest that if oligodendrocyte precursor cells in the multiple sclerosis spinal cord express NG2, they do so at much lower levels than oligodendrocyte precursor cells in brain lesions and non-myelinating Schwann cells in spinal nerves. No convincing labelling of oligodendrocyte precursor-like cells in the spinal cord sections was obtained with the R7 polyclonal antibody to the C-terminus of the human PDGF- α receptor (Claesson-Welsh *et al.*, 1989), which was also used in the study of Scolding and co-workers (Scolding *et al.*, 1998); using the

Table 3 *Densities of oligodendrocyte precursor cells, immature oligodendrocytes and phase-bright macrophages in demyelinated spinal cord and brain lesions*

MS case	Number of cells/mm ²		
	O4-positive, GalC-negative	O4-positive GalC-positive	Phase-bright macrophages
Spinal cord lesions			
1*	10.6 \pm 1.3	0.2 \pm 0.4	0.2 \pm 0.4
2*	33.2 \pm 5.6	4.2 \pm 2.5	245.0 \pm 37.3
3	34.8 \pm 1.8	3.5 \pm 1.8	1.4 \pm 1.8
4	1.6 \pm 1.0	<0.2	0.3 \pm 0.3
5	12.9 \pm 0.4	<0.2	5.2 \pm 5.9
6	1.7 \pm 2.6	<0.2	2.0 \pm 0.9
7a†	10.0 \pm 4.3	0.3 \pm 0.5	37.3 \pm 16.6
7b	9.5 \pm 7.1	0.2 \pm 0.3	1.7 \pm 0.7
8	<0.2	<0.2	<0.2
9	19.2 \pm 7.6	0.9 \pm 0.6	164.6 \pm 14.7
10	9.1 \pm 2.3	<0.2	<0.2
11	<0.3	<0.3	<0.3
12	3.0 \pm 1.9	<0.2	<0.2
13	1.0 \pm 1.7	<0.3	10.7 \pm 7.3
14	10.8 \pm 5.7	<0.2	41.7 \pm 4.6
15	10.5 \pm 2.1	<0.2	2.5 \pm 0.9
16	16.7 \pm 0.8	<0.2	<0.2
Brain lesions			
7	19.6 \pm 0.7	0.2 \pm 0.3	32.9 \pm 7.2
8	11.7 \pm 2.0	<0.2	0.7 \pm 1.2
10	13.0 \pm 2.6	0.5 \pm 0.6	0.6 \pm 0.6
11	8.3 \pm 1.3	<0.2	2.7 \pm 1.4
13	25.6 \pm 4.4	0.2 \pm 0.3	25.1 \pm 18.9
14a‡	38.4 \pm 3.8	4.3 \pm 1.1	188.1 \pm 9.3
14b	12.6 \pm 4.8	0.2 \pm 0.3	10.0 \pm 6.1
15	10.8 \pm 4.1	<0.2	3.0 \pm 1.7
16	16.8 \pm 1.8	<0.3	4.8 \pm 3.0

*Brain lesions from multiple sclerosis cases 1 and 2 were examined in a previous study (Wolswijk, 1998b). †Two different spinal cord samples (a and b) from multiple sclerosis subject 7 were analysed. ‡Two different brain lesions (a and b) from multiple sclerosis case 14 were analysed.

same immunolabelling procedure, these antibodies did label reproducibly process-bearing, oligodendrocyte precursor-like cells in sections of marmoset and rhesus monkey brain tissue (G. Wolswijk, B. 't Hart and H. Brok, unpublished observations).

O4-positive, GalC-negative cells were observed throughout the demyelinated areas of most spinal cord samples, including the affected grey matter areas (Table 3). They had an oval-shaped cell body containing an oval or irregular-shaped nucleus and little cytoplasm (Fig. 2). The small number of processes (1–4) that emanated from their cell body were fine, and sometimes long (Fig. 1); an occasional O4-positive, GalC-negative cell had a more elongated morphology. These cells were in close proximity to numerous demyelinated axons and were embedded in an often dense network of GFAP-containing astrocytic processes (Figs. 1 and 2).

The highest numbers of O4-positive, GalC-negative cells were observed in the demyelinated areas of the spinal cord

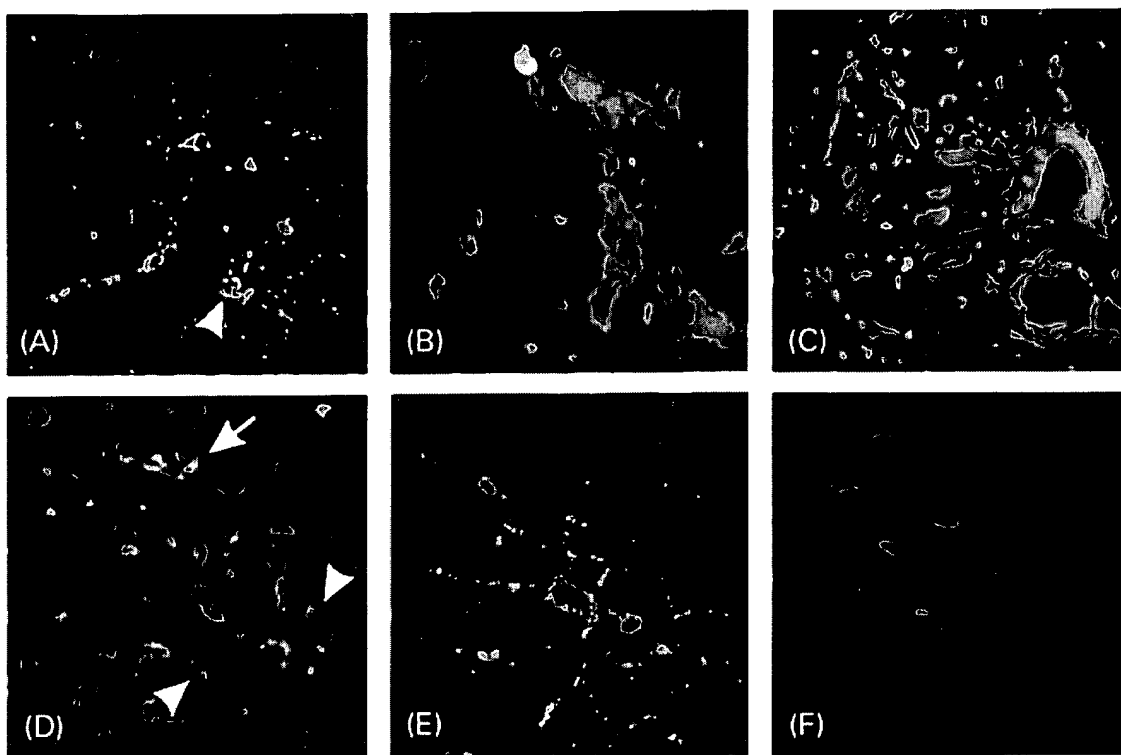


Fig. 2 Details of oligodendrocyte precursor cells in demyelinated spinal cord lesions. (A) Two O4-positive (green, FITC), GalC-negative (blue, Cy5) oligodendrocyte precursor cells in an area with large numbers of neurofilament-positive (red, TRITC), demyelinated axons. The arrowhead points to a GalC-positive process that has wrapped a denuded axon; the oligodendrocyte to which this process belonged was in another section. Multiple sclerosis case 9. Image size, $135\ \mu\text{m} \times 135\ \mu\text{m}$. (B) Oligodendrocyte precursor cell that had bound the O4 antibody (red, TRITC), but not antibodies to GalC (blue, Cy5), in an area with macrophages expressing HLA-DP, DQ, DR antigens (green, FITC). Multiple sclerosis case 14. Image size, $45\ \mu\text{m} \times 45\ \mu\text{m}$. (C) O4-positive, GalC-negative oligodendrocyte precursor cells appear to lack the astrocyte-specific intermediate filament GFAP, as shown previously for O4-positive, GalC-negative cells in brain lesions (Wolswijk, 1998b). The expression of GFAP in most demyelinated areas of the spinal cords studied was much more pronounced than in the image shown here (see Fig. 1E) (O4 = red, TRITC; GalC = blue, Cy5; GFAP = green, FITC). Multiple sclerosis case 5. Image size, $50\ \mu\text{m} \times 50\ \mu\text{m}$. (D) Some processes of the GalC-positive cell (red, TRITC) shown were connected to MBP-positive (green, FITC) myelin sheaths (arrow), while others had encircled individual neurofilament-positive (blue, Cy5) axons, but had not formed myelin (arrowheads). The cell body of this, presumably immature, oligodendrocyte contained some MBP immunoreactivity. Multiple sclerosis case 2. Image size, $105\ \mu\text{m} \times 105\ \mu\text{m}$. (E) NG2-positive (green, FITC) oligodendrocyte precursor cell in a brain lesion from multiple sclerosis case 13. The cell shown was present in a demyelinated area containing many HLA-DP, DQ, DR-positive macrophages (red, TRITC); nuclei were visualized with the nuclear dye TO-PRO-3 iodide (blue). Although NG2-positive cells were observed in a brain lesion from multiple sclerosis case 13, no NG2-positive cells were observed in the spinal cord sample from this subject. However, the antibodies to NG2 did label cells in spinal nerve tissue from all multiple sclerosis subjects (see Fig. 2F). Image size, $90\ \mu\text{m} \times 90\ \mu\text{m}$. (F) NG2-positive cell (red, Cy3) in a spinal nerve attached to the spinal cord sample obtained from multiple sclerosis case 8; nuclei in the section were visualized with TO-PRO-3 iodide (blue). A recent study has suggested that NG2-positive cells in adult rat peripheral nerves are non-myelinating Schwann cells (Schneider *et al.*, 2001). Image size, $80\ \mu\text{m} \times 80\ \mu\text{m}$.

sections from multiple sclerosis cases 2 and 3 ($33\text{--}35$ cells/ mm^2 section; $10\ \mu\text{m}$ thick sections) (Table 3 and Fig. 3), with up to seven cells/microscope field (size: $1/16\ \text{mm}^2$). Although both samples harboured comparable numbers of O4-positive GalC-negative cells, the spinal cord lesion from multiple sclerosis case 2 contained many phase-bright macrophages (245.0 ± 37.3 cells/ mm^2), while the spinal cord lesion from multiple sclerosis case 3 virtually lacked macrophages (Table 3 and Fig. 3), suggesting it was a relatively old lesion; it has been suggested that it can take >6 months for debris-laden macrophages to disappear from demyelinated areas (Brück *et al.*, 1995). Eight of the spinal cord samples contained

between 10 and 20 O4-positive, GalC-negative cells/ mm^2 , while oligodendrocyte precursor cells were rare (≤ 3.0 cells/ mm^2) in the demyelinated samples from multiple sclerosis cases 4, 6, 8, 11, 12 and 13 (Table 3). Oligodendrocyte precursor cells were detected in demyelinated spinal cord tissue derived from both subjects with PP multiple sclerosis (9.1 ± 2.3 and 10.6 ± 1.3 cells/ mm^2) and from six out of seven subjects with SP multiple sclerosis (up to 34.8 ± 1.8 cells/ mm^2) (Table 3).

Statistical analysis indicated that there was a significant negative correlation between the density of O4-positive, GalC-negative precursor cells in the demyelinated spinal cord

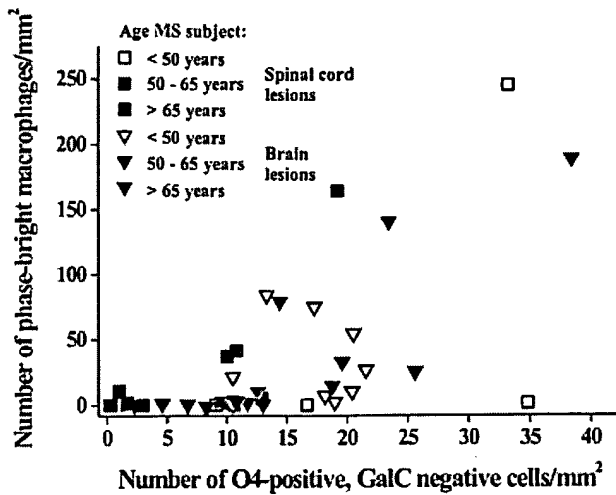


Fig. 3 There is a positive correlation between the density of the oligodendrocyte precursor population and the macrophage population in spinal cord and brain lesions from subjects with chronic multiple sclerosis. The data suggest that the number of O4-positive, GalC-negative oligodendrocyte precursor cells/mm² section was higher in lesions containing numerous macrophages than in lesions with only small numbers of macrophages [data from 17 spinal cord (squares) and 24 brain lesions (triangles)]; 15 brain lesions were analysed in a previous study (Wolswijk, 1998b; see Table 1 for details of these multiple sclerosis cases). A clear exception was the spinal cord sample with demyelination obtained from Case 3, which had one of the highest precursor densities (35 ± 2 cells/mm²), but virtually lacked macrophages (<3 cells/mm²). Since a higher proportion of the lesions obtained from the older multiple sclerosis subjects, who also had a longer disease duration, lacked macrophages, there was a negative correlation between the density of the O4-positive, GalC-negative oligodendrocyte precursor population and the age of the multiple sclerosis subject at death, and length of the disease process. MS = multiple sclerosis.

areas, and the age of the multiple sclerosis subject at death ($P = 0.0002$) (Fig. 3) and clinical disease duration ($P = 0.006$); as expected, the length of the disease process increased significantly with age of the multiple sclerosis subject ($P = 0.004$). Moreover, there was a positive correlation between the density of the macrophage population, which gives an indication of the relative age of the lesion (Ozawa *et al.*, 1994; Brück *et al.*, 1995), and the density of the oligodendrocyte precursor cell population ($P = 0.0132$), suggesting that lesions with few if any macrophages, i.e. relatively old lesions, contained fewer O4-positive, GalC-negative cells than lesions with numerous macrophages, i.e. relatively fresh lesions. A clear exception was the spinal cord sample from multiple sclerosis case 3, which harboured many O4-positive, GalC-negative cells, but lacked macrophages. The analysis further showed that spinal cord lesions with only small numbers of macrophages (<10 cells/mm²) were most often derived from subjects over the age of 65 (Fig. 3).

Immature GalC-positive oligodendrocytes were rare in the demyelinated areas of most chronic multiple sclerosis spinal

cord samples (<0.5 cell/mm² section). The three exceptions were those derived from multiple sclerosis cases 2 (4.2 ± 2.5 cells/mm²), 3 (3.5 ± 1.8 cells/mm²) and 9 (0.9 ± 0.6 cells/mm²); these lesions also harboured the highest numbers of O4-positive, GalC-negative cells/mm² section (Table 3). These presumably newly generated oligodendrocytes tended to be present in clusters [with up to four cells/microscope field ($1/16$ mm²)] and these clusters also contained O4-positive, GalC-negative cells (Fig. 2). The processes of some of the GalC-positive cells had encircled individual denuded axons in the lesion area and/or were connected to MBP-positive myelin sheaths (Fig. 2).

Oligodendrocyte precursor cells in brain lesions

Appropriately fixed brain lesions were available from three of the six chronic multiple sclerosis cases with no or only small numbers of O4-positive, GalC-negative cells in their spinal cord lesions (multiple sclerosis cases 8, 11 and 13). Immunolabellings demonstrated that these brain lesions retained numerous O4-positive, GalC-negative cells (8.3 ± 1.3 , 11.7 ± 2.0 and 25.6 ± 4.4 cells/mm²) (Table 3). These cells were also abundant in demyelinated brain lesions analysed from some of the other multiple sclerosis cases ($n = 7$; range 10.5 ± 2.0 to 38.4 ± 3.8 cells/mm²; Table 3). GalC-positive cells with an immature morphology were present in only very small numbers in the brain lesions (Table 3), as shown previously (Wolswijk, 2000). The highest density was observed in a brain lesion from multiple sclerosis case 14 (4.3 ± 1.1 cells/mm²), which also contained the highest density of O4-positive, GalC-negative cells (38.4 ± 3.8 cells/mm²) and phase-bright macrophages (188.1 ± 9.3 cells/mm²) (Table 3).

Factors influencing the density of the oligodendrocyte precursor population in multiple sclerosis lesions

The combined results from the spinal cord and brain lesions (41 lesions in total) analysed in the present study and previously [Wolswijk, 1998b; see Table 1 for details of the multiple sclerosis subjects whose brain lesions ($n = 15$) were studied previously] further supported the indications that the density of the O4-positive, GalC-negative cells in the lesion areas gradually decreased with increasing age of the multiple sclerosis subject ($P < 0.0001$) (Fig. 3), length of the disease process ($P = 0.006$) and with declining numbers of macrophages ($P < 0.0001$). Moreover, the density of the macrophage populations in the lesions also decreased with age of the multiple sclerosis subject at death ($P = 0.040$) (see Fig. 3). Furthermore, the density of the O4-positive, GalC-negative cell population (and macrophage population) in lesions ($n = 5$) derived from subjects with the PP form of multiple sclerosis ($n = 4$) was not significantly different from that in lesions ($n = 16$) derived from subjects with the SP form

of multiple sclerosis ($n = 12$) [12.0 ± 7.6 versus 16.5 ± 8.8 O4-positive, GalC-negative cells/mm² (and 28.8 ± 62.3 versus 47.9 ± 69.6 macrophages/mm²)].

Discussion

The present study establishes for the first time that O4-positive, GalC-negative oligodendrocyte precursors are abundant in demyelinated spinal cord lesions from most subjects with longstanding multiple sclerosis ($n = 16$), including those with the PP and SP form, with densities of up to 35 cells/mm². The present findings thus extend those of previous histopathological studies demonstrating the presence of a sizeable population of oligodendrocyte precursor cells in demyelinated brain lesions from subjects with chronic multiple sclerosis, identified using either the O4/anti-GalC antibody combination (Wolswijk, 1998b; 14 multiple sclerosis cases; range of densities 2–34 cells/mm²), antibodies to the NG2 chondroitin sulphate proteoglycan [Chang *et al.*, 2000; five multiple sclerosis cases; densities were only reported for three inactive lesions from a single chronic multiple sclerosis case (40–80 NG2-positive cells/mm² in 30 μ m thick sections, which corresponds to a density of 13–27 cells/mm² cells in 10 μ m thick sections)] or antibodies to the PDGF- α receptor (Scolding *et al.*, 1998; five multiple sclerosis cases; range of densities 1–3 PDGF- α receptor-positive cells/100 nuclei). A recent study has reported, however, that brain lesions often contain much higher numbers of PDGF- α receptor-positive cells and that many of these cells are not oligodendrocyte precursor cells, but either oligodendrocytes or astrocytes (Maeda *et al.*, 2001).

Analysis of the density of O4-positive, GalC-negative oligodendrocyte precursor cells in demyelinated spinal cord ($n = 17$) and brain lesions ($n = 24$) from 26 subjects with chronic multiple sclerosis suggests that their density decreased significantly with increasing age of the multiple sclerosis subject, duration of clinical symptoms and increasing age of the lesion, as judged by the presence and number of macrophages filled with myelin degradation products. The factor most likely to influence the density of the oligodendrocyte precursor population in the lesions is probably the age of the lesion, because, as would be expected, lesions with only small numbers of macrophages were derived most commonly from the older subjects with a long duration of clinical symptoms. If this is indeed the case, it suggests that O4-positive, GalC-negative precursor cells slowly disappear from demyelinated areas with lesion progression, possibly due to diminishing amounts of appropriate survival factors. Moreover, the presence of only small numbers of oligodendrocyte precursor cells in relatively old lesions suggests that migration of precursor cells from unaffected spinal cord regions into lesion areas is limited, in contrast to what is observed in some models of CNS demyelination (Franklin *et al.*, 1997; Keirstead *et al.*, 1998). Repeated damage may also play an important role in the depletion of the oligodendrocyte precursor pool, as suggested by experimen-

tal studies (Keirstead *et al.*, 1998). The finding that significant numbers of immature oligodendrocytes were only present in lesions with high precursor densities suggests that the ability of precursor cells to differentiate becomes increasingly more impaired with lesion evolution. Although some studies have provided evidence that lesions in the CNS of patients with PP multiple sclerosis differ in some aspects from those present in the CNS of patients with the SP form (e.g. Revesz *et al.*, 1994; Lycklama à Nijeholt *et al.*, 2001), no significant difference in oligodendrocyte precursor densities was found between lesions from these patient groups.

Demyelinated lesions with no or only few O4-positive, GalC-negative oligodendrocyte precursor cells (≤ 3.0 cells/mm²) were more common in the spinal cord than in the brain of subjects with longstanding multiple sclerosis analysed thus far [six out of 17 spinal cord lesions (35%) versus one out of 24 brain lesions (4%) studied; Wolswijk, 1998b; present study]. This difference appeared not to be patient-specific, as immunolabellings of brain lesions from three of the six multiple sclerosis subjects with low precursor densities that were available for study did contain numerous O4-positive, GalC-negative cells (8–26 cells/mm²). Since a higher proportion of the brain lesions contained ≥ 10 phase-bright macrophages/mm² than the spinal cord lesions [54% (13 out of 24) versus 29% (five out of 17)], it suggests that the spinal cord lesions analysed were on average older than the brain lesions studied, and, because of this, frequently harboured only small numbers of O4-positive, GalC-negative precursor cells.

As reported previously (Wolswijk, 1998b), the O4/anti-GalC antibody combination was not useful for the detection of O4-positive, GalC-negative oligodendrocyte precursor cells in areas with large numbers of O4-positive, GalC-positive oligodendrocytes and myelin sheaths, and it was thus not possible to assess the density of the precursor population in control and unaffected multiple sclerosis spinal cord tissue. It was thus also not possible to determine whether the density of the oligodendrocyte precursor population decreases significantly with age. However, studies in the rat have indicated that up to 8% of cells in the adult rat CNS are oligodendrocyte precursor cells (Dawson *et al.*, 2000; Levine *et al.*, 2001). If this is also true for the human CNS, it suggests that up to 37 ± 4 cells/mm² in the adult human spinal cord are oligodendrocyte precursor cells [spinal cord white matter from three subjects without neurological disease (54 ± 21 years of age) contained 463 ± 50 nuclei/mm² (10 μ m thick sections)]. This estimate corresponds very well with that reported for the intact adult rat spinal cord (McTigue *et al.*, 2001). Furthermore, Chang *et al.* (2000) found that between 140 and 150 cells/mm² were NG2-positive in the white matter surrounding three inactive brain lesions derived from a single chronic multiple sclerosis case (30 μ m thick sections; this corresponds to a density of 47–50 cells/mm² in a 10 μ m section). These figures are very similar to the highest density for O4-positive, GalC-negative oligodendrocyte precursor cells found in both brain (38 cells/mm²) and spinal cord

lesions (35 cells/mm²), suggesting that death of precursor cells during the actual myelin destruction phase in many multiple sclerosis cases may be limited. Instead, the data suggest that the size of the oligodendrocyte precursor population gradually decreases with advancing age of the lesion. However, there are clearly some exceptions. For example, one of the two spinal cord lesions with the highest density of precursor cells completely lacked macrophages (Table 3), while two distinct regions of a brain lesion studied previously (Wolswijk, 1998b) harboured comparable numbers of oligodendrocyte precursor cells, but one area was devoid of macrophages, while the other contained numerous macrophages laden with myelin degradation products (Wolswijk, 1998b). These findings thus suggest that the number of oligodendrocyte precursor cells in some lesions may remain high for prolonged periods of time.

Complete destruction or severe depletion of the oligodendrocyte precursor population may occur in some cases of multiple sclerosis. This indication has come from the study of Chang *et al.* (2000) who found that two actively demyelinating brain lesions derived from two multiple sclerosis subjects with short clinical duration (<1 year) completely lacked NG2-positive cells. Oligodendrocyte precursor cells may die as a result of non-specific mechanisms or of a specific immunological response to a molecule expressed on the surface of oligodendrocyte precursor cells or to a surface molecule that these cells share with oligodendrocytes and myelin [e.g. the antigen(s) recognized by the O4 antibody]. In this respect, it is interesting to note that Niehaus and colleagues found that patients with active RR multiple sclerosis synthesize antibodies recognizing a protein expressed on the surface of rat oligodendrocyte precursor cells (Niehaus *et al.*, 2000), a molecule which appears to be homologous to NG2 (Diers-Fenger *et al.*, 2001). Thus, it is possible that there are distinct multiple sclerosis subtypes with respect to oligodendrocyte precursor survival and destruction, as appears to be the case for patterns of oligodendrocyte pathology (Lucchinetti *et al.*, 2000). To gain further insights into this issue, it will be necessary to analyse in detail actively demyelinating lesions from both acute and chronic multiple sclerosis cases, but this material unfortunately is rare.

Oligodendrocyte precursor cells in multiple sclerosis lesions and control human CNS tissue have been identified using different markers. NG2-positive cells in the control adult human (and rodent) CNS display a highly complex morphology that is distinct from that of ramified microglia, astrocytes and myelinating oligodendrocytes (Levine *et al.*, 1993; Nishiyama *et al.*, 1996; Reynolds and Hardy, 1997; Oumesmar *et al.*, 1997; Chang *et al.*, 2000; Dawson *et al.*, 2000). The results obtained with antibodies to the PDGF- α receptor are more confusing. Chang *et al.* (2000) reported that PDGF- α receptor-expressing cells in the human CNS express a morphology that is very similar to that of NG2-positive cells, as do PDGF- α receptor-positive cells in the marmoset and rhesus monkey CNS (G. Wolswijk, B. 't Hart and H. Brok, unpublished observations). In contrast, Scolding *et al.*

(1998) found that such cells were either round or bipolar, while Maeda *et al.* (2001) found that antibodies to the PDGF- α receptor labelled the cell body of mature, 2',3'-CNPase (cyclic nucleotide phosphohydrolase)-expressing oligodendrocytes in control human brain white matter. O4-positive, GalC-negative cells in multiple sclerosis lesions are process bearing and resemble morphologically those expressing NG2 [compare images provided in Wolswijk (1998b); Chang *et al.* (2000) and in the present study; see also Dawson *et al.* (2000)], although some NG2-positive cells in some lesions appear to express a more elongated morphology (Chang *et al.*, 2000). PDGF- α receptor-positive cells in multiple sclerosis lesions have been reported to express a rounded or bipolar morphology, with none expressing the oligodendrocyte markers GalC or Rip and astrocyte marker GFAP (Scolding *et al.*, 1998), while another study presented data indicating that cells expressing the PDGF- α receptor in lesion areas frequently express CNPase or GFAP (Maeda *et al.*, 2001), suggesting that they are either oligodendrocytes or astrocytes, respectively. Clearly, further studies are needed to clarify the identity of the various antigenically identified populations and to determine whether there are antigenically and morphologically distinct subsets of oligodendrocyte precursor cells. That some overlap may exist is suggested by the observations that NG2-positive cells in the cerebral cortex of the adult rat bind the O4 antibody (Reynolds and Hardy, 1997), that NG2-positive cells in the developing rat CNS express the PDGF- α receptor (Nishiyama *et al.*, 1996) and that oligodendrocyte precursor cells freshly isolated from adult rat optic nerves and spinal cords bind the O4 antibody and divide *in vitro* in response to PDGF (Wolswijk *et al.*, 1991; Wolswijk and Noble, 1992; Engel and Wolswijk, 1996; Shi *et al.*, 1998), suggesting that the O4-positive cells have receptors for PDGF. Indeed, *in situ* hybridization experiments have shown that cultured O4-positive cells from the adult human CNS contain transcripts for the PDGF- α receptor (Gogate *et al.*, 1994).

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References

- Alonso G. Prolonged corticosterone treatment of adult rats inhibits the proliferation of oligodendrocyte progenitors present throughout white and gray matter regions of the brain. *Glia* 2000; 31: 219–31.
- Arenella LS, Herndon RM. Mature oligodendrocytes. Division following experimental demyelination in adult animals. *Arch Neurol* 1984; 41: 1162–5.
- Back SA, Luo NL, Borenstein NS, Levine JM, Volpe JJ, Kinney HC. Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. *J Neurosci* 2001; 21: 1302–12.
- Brück W, Porada P, Poser S, Rieckmann P, Hanefeld F, Kretzschmar HA, et al. Monocyte/macrophage differentiation in early multiple sclerosis lesions. *Ann Neurol* 1995; 38: 788–96.
- Carroll WM, Jennings AR. Early recruitment of oligodendrocyte precursors in CNS demyelination. *Brain* 1994; 117: 563–78.
- Chang A, Nishiyama A, Peterson J, Prineas J, Trapp BD. NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. *J Neurosci* 2000; 20: 6404–12.
- Claesson-Welsh L, Eriksson A, Westermarck B, Heldin CH. cDNA cloning and expression of the human A-type platelet-derived growth factor (PDGF) receptor establishes structural similarity to the B-type PDGF receptor. *Proc Natl Acad Sci USA* 1989; 86: 4917–21.
- Dawson MR, Levine JM, Reynolds R. NG2-expressing cells in the central nervous system: are they oligodendroglial progenitors? [Review]. *J Neurosci Res* 2000; 61: 471–9.
- Di Bello IC, Dawson MR, Levine JM, Reynolds R. Generation of oligodendroglial progenitors in acute inflammatory demyelinating lesions of the rat brain stem is associated with demyelination rather than inflammation. *J Neurocytol* 1999; 28: 365–81.
- Diers-Fenger M, Kirchhoff F, Kettenmann H, Levine JM, Trotter J. AN2/NG2 protein-expressing glial progenitor cells in the murine CNS: isolation, differentiation, and association with radial glia. *Glia* 2001; 34: 213–28.
- Engel U, Wolswijk G. Oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells derived from adult rat spinal cord: in vitro characteristics and response to PDGF, bFGF and NT-3. *Glia* 1996; 16: 16–26.
- Franklin RJ. Remyelination—a regenerative process in the CNS. [Review]. *Neuroscientist* 1999; 5: 184–91.
- Franklin RJ, Gilson JM, Blakemore WF. Local recruitment of remyelinating cells in the repair of demyelination in the central nervous system. *J Neurosci Res* 1997; 50: 337–44.
- Gensert JM, Goldman JE. Endogenous progenitors remyelinate demyelinated axons in the adult CNS. *Neuron* 1997; 19: 197–203.
- Gilson J, Blakemore WF. Failure of remyelination in areas of demyelination produced in the spinal cord of old rats. *Neuropathol Appl Neurobiol* 1993; 19: 173–81.
- Godfraind C, Friedrich VL, Holmes KV, Dubois-Dalcq M. In vivo analysis of glial cell phenotypes during a viral demyelinating disease in mice. *J Cell Biol* 1989; 109: 2405–16.
- Gogate N, Verma L, Zhou JM, Milward E, Rusten R, O'Connor M, et al. Plasticity in the adult human oligodendrocyte lineage. *J Neurosci* 1994; 14: 4571–87.
- Hinks GL, Franklin RJ. Distinctive patterns of PDGF-A, FGF-2, IGF-I, and TGF-beta1 gene expression during remyelination of experimentally-induced spinal cord demyelination. *Mol Cell Neurosci* 1999; 14: 153–68.
- Jeffery ND, Blakemore WF. Locomotor deficits induced by experimental spinal cord demyelination are abolished by spontaneous remyelination. *Brain* 1997; 120: 27–37.
- Keirstead HS, Levine JM, Blakemore WF. Response of the oligodendrocyte progenitor cell population (defined by NG2 labelling) to demyelination of the adult spinal cord. *Glia* 1998; 22: 161–70.
- Komoly S, Hudson LD, Webster HD, Bondy CA. Insulin-like growth factor I gene expression is induced in astrocytes during experimental demyelination. *Proc Natl Acad Sci USA* 1992; 89: 1894–8.
- Lassmann H, Brück W, Lucchinetti C, Rodriguez M. Remyelination in multiple sclerosis. [Review]. *Mult Scler* 1997; 3: 133–6.
- Levine JM, Stincone F, Lee Y-S. Development and differentiation of glial precursor cells in the rat cerebellum. *Glia* 1993; 7: 307–21.
- Levine JM, Reynolds R, Fawcett JW. The oligodendrocyte precursor cell in health and disease. [Review]. *Trends Neurosci* 2001; 24: 39–47.
- Lucchinetti C, Brück W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol* 2000; 47: 707–17.
- Ludwin SK. An autoradiographic study of cellular proliferation in remyelination of the central nervous system. *Am J Pathol* 1979; 95: 683–96.
- Ludwin SK. Pathology of demyelination and remyelination. [Review]. In: Waxman SG, Ritchie JM, editors. *Demyelinating diseases: basic and clinical electrophysiology*. Advances in neurology, Vol. 31. New York: Raven Press; 1981. p. 123–168.
- Lycklama à Nijeholt GJ, Bergers E, Kamphorst W, Bot J, Nicolay K, Castelijns JA, et al. Post-mortem high-resolution MRI of the spinal cord in multiple sclerosis: a correlative study with conventional MRI, histopathology and clinical phenotype. *Brain* 2001; 124: 154–66.
- Maeda Y, Solanky M, Menonna J, Chapin J, Li W, Dowling P. Platelet-derived growth factor- α receptor-positive oligodendroglia are frequent in multiple sclerosis lesions. *Ann Neurol* 2001; 49: 776–85.
- McTigue DM, Wei P, Stokes BT. Proliferation of NG2-positive cells and altered oligodendrocyte numbers in the contused rat spinal cord. *J Neurosci* 2001; 21: 3392–400.

- Messersmith DJ, Murtie JC, Le TQ, Frost EE, Armstrong RC. Fibroblast growth factor 2 (FGF2) and FGF receptor expression in an experimental demyelinating disease with extensive remyelination. *J Neurosci Res* 2000; 62: 241–56.
- Niehaus A, Shi J, Grzenkowski M, Diers-Fenger M, Hartung HP, et al. Patients with active relapsing–remitting multiple sclerosis synthesize antibodies recognizing oligodendrocyte progenitor cell surface protein: implications for remyelination. *Ann Neurol* 2000; 48: 362–71.
- Nishiyama A, Lin XH, Giese N, Heldin CH, Stallcup WB. Co-localization of NG2 proteoglycan and PDGF alpha-receptor on O2A progenitor cells in the developing rat brain. *J Neurosci Res* 1996; 43: 299–314.
- Oumesmar BN, Vignais L, Baron-van Evercooren A. Developmental expression of platelet-derived growth factor α -receptor in neurons and glial cells of the mouse CNS. *J Neurosci* 1997; 17: 125–39.
- Ozawa K, Suchanek G, Breitschopf H, Brück W, Budka H, Jellinger K, et al. Patterns of oligodendroglia pathology in multiple sclerosis. *Brain* 1994; 117: 1311–22.
- Piddlesden SJ, Lassmann H, Zimprich F, Morgan BP, Linington C. The demyelinating potential of antibodies to myelin oligodendrocyte glycoprotein is related to their ability to fix complement. *Am J Pathol* 1993; 143: 555–64.
- Prineas JW, McDonald WI. Demyelinating diseases. [Review]. In: Graham DI, Lantos PL, editors. *Greenfield's neuropathology*. 6th edn. London: Arnold; 1997. p. 813–96.
- Prineas JW, Kwon EE, Goldenberg PZ, Ilyas AA, Quarles RH, Benjamins JA, et al. Multiple sclerosis. Oligodendrocyte proliferation and differentiation in fresh lesions. *Lab Invest* 1989; 61: 489–503.
- Raine CS, Wu E. Multiple sclerosis: remyelination in acute lesions. *J Neuropathol Exp Neurol* 1993; 52: 199–204.
- Ranscht B, Clapshaw PA, Price J, Noble M, Seifert W. Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. *Proc Natl Acad Sci USA* 1982; 79: 2709–13.
- Redwine JM, Armstrong RC. In vivo proliferation of oligodendrocyte progenitors expressing PDGFalphaR during early remyelination. *J Neurobiol* 1998; 37: 413–28.
- Revesz T, Kidd D, Thompson AJ, Barnard RO, McDonald WI. A comparison of the pathology of primary and secondary progressive multiple sclerosis. *Brain* 1994; 117: 759–65.
- Reynolds R, Hardy R. Oligodendroglial progenitors labeled with the O4 antibody persist in the adult rat cerebral cortex in vivo. *J Neurosci Res* 1997; 47: 455–70.
- Rodriguez M. Immunoglobulins stimulate central nervous system remyelination: electron microscopic and morphometric analysis of proliferating cells. *Lab Invest* 1991; 64: 358–70.
- Schneider S, Bosse F, D'Urso D, Muller H, Sereda MW, Nave K, et al. The AN2 protein is a novel marker for the Schwann cell lineage expressed by immature and nonmyelinating Schwann cells. *J Neurosci* 2001; 21: 920–33.
- Scolding N, Franklin R, Stevens S, Heldin CH, Compston A, Newcombe J. Oligodendrocyte progenitors are present in the normal adult human CNS and in the lesions of multiple sclerosis. *Brain* 1998; 121: 2221–8.
- Shi J, Marinovich A, Barres BA. Purification and characterization of adult oligodendrocyte precursor cells from the rat optic nerve. *J Neurosci* 1998; 18: 4627–36.
- Shields SA, Gilson JM, Blakemore WF, Franklin RJ. Remyelination occurs as extensively but more slowly in old rats compared to young rats following gliotoxin-induced CNS demyelination. *Glia* 1999; 28: 77–83.
- Smith KJ. Conduction properties of central demyelinated axons: the generation of symptoms in demyelinating disease. [Review]. In: Bostock H, Kirkwood PA, Pullen AH, editors. *The neurobiology of disease*. Cambridge: Cambridge University Press; 1996. p. 95–117.
- Sommer I, Schachner M. Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. *Dev Biol* 1981; 83: 311–27.
- Tourbah A, Baron-Van Evercooren A, Oliver L, Raulais D, Jeanny JC, Gumpel M. Endogenous aFGF expression and cellular changes after a demyelinating lesion in the spinal cord of adult normal mice: immunohistochemical study. *J Neurosci Res* 1992; 33: 47–59.
- Wingerchuk DM, Lucchinetti CF, Noseworthy JH. Multiple sclerosis: current pathophysiological concepts. [Review]. *Lab Invest* 2001; 81: 263–81.
- Wolswijk G. Oligodendrocyte regeneration in the adult rodent CNS and the failure of this process in multiple sclerosis. [Review]. *Prog Brain Res* 1998a; 117: 233–47.
- Wolswijk G. Chronic stage multiple sclerosis lesions contain a relatively quiescent population of oligodendrocyte precursor cells. *J Neurosci* 1998b; 18: 601–9.
- Wolswijk G. Oligodendrocyte survival, loss and birth in lesions of chronic-stage multiple sclerosis. *Brain* 2000; 123: 105–15.
- Wolswijk G, Noble M. Cooperation between PDGF and FGF converts slowly dividing O-2A^{adult} progenitor cells to rapidly dividing cells with characteristics of O-2A^{perinatal} progenitor cells. *J Cell Biol* 1992; 118: 889–900.
- Wolswijk G, Riddle PN, Noble M. Platelet-derived growth factor is mitogenic for O-2A^{adult} progenitor cells. *Glia* 1991; 4: 495–503.
- Wood JN, Anderton BH. Monoclonal antibodies to mammalian neurofilaments. *Biosci Rep* 1981; 1: 263–8.
- Yao DL, West NR, Bondy CA, Brenner M, Hudson LD, Zhou J, et al. Cryogenic spinal cord injury induces astrocytic gene expression of insulin-like growth factor I and insulin-like growth factor binding protein 2 during myelin regeneration. *J Neurosci Res* 1995; 40: 647–59.

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The use of animal models to investigate the pathogenesis of neuroinflammatory disorders of the central nervous system

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Purpose of review

A major hurdle in the research of neuroinflammatory disorders of the central nervous system is the inaccessibility of the organ. Thus investigation is limited to end-stage disease and systemic changes that occur during disease progression, neither of which may reflect the pathological process in the central nervous system. These factors may explain the lack of effective therapies in multiple sclerosis, a common chronic inflammatory disease of the central nervous system. To overcome such limitations approaches using more relevant animal models have been developed to study pathological mechanisms as well as the design of rational therapeutic strategies. This review describes the animal models used to study pathological processes leading to inflammation within the central nervous system that may be operating in multiple sclerosis and the use of these models in the design of more rational therapeutic strategies.

Recent findings

The clinical heterogeneity of multiple sclerosis as well as the finding of different pathological patterns suggests that multiple sclerosis may be a spectrum of diseases that may represent different pathological processes. This and the renewed interest in the extent of axonal damage has led to the development of more relevant animal models, such as those in nonhuman primates, that both reflect the spectrum of multiple sclerosis and allow the development of species-specific therapeutic approaches.

Summary

While many animal models are available, the use of relevant animal models that mimic either the different forms of multiple sclerosis or the spectrum of multiple sclerosis is critical to examine those factors, for example genes or proteins, that are of pathogenic relevance and can be used as targets for therapy.

Keywords

multiple sclerosis, animal models, therapy

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Abbreviations

APC	antigen-presenting cell
BBB	blood–brain barrier
CSF	cerebrospinal fluid
CNS	central nervous system
EAE	experimental autoimmune encephalomyelitis
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
SFV	Semliki-Forest virus
TMEV	Theiler's murine encephalomyelitis virus

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Introduction

With an incidence of one per 1000 affected young adults, multiple sclerosis (MS) is the most prevalent inflammatory disease of the human central nervous system (CNS). The pathological hallmark of MS is the lesion, a focally demyelinated area within CNS white matter and cortex with a variable degree of inflammation, gliosis, axonal pathology and remyelination [1]. In a large multicenter trial at least four fundamentally different patterns of pathology were distinguished [2]. Two patterns show close similarity with the pathology in the animal model experimental autoimmune encephalomyelitis (EAE), where lesions are induced by autoreactive T cells alone (pattern I) or T cells plus autoantibodies (pattern II). Patterns III and IV do not seem to be of immune origin, but rather formed by oligodendrocyte dystrophy. That the pathological features of lesions differ between patients, but are homogeneous within a patient, suggests different underlying pathogenic mechanisms. This has obvious consequences for therapy development.

The factor that triggers the anti-myelin autoimmune reaction in genetically susceptible individuals is likely to be the activation of preexisting myelin reactive T and B cells by a microbial infection [3]. Recent findings from genome-wide screens confirm earlier data that an important genetic influence is exerted by the major histocompatibility complex (MHC) [4]. This highly polymorphic genomic region located on chromosome 6 encodes the HLA class I and II molecules that present antigen determinants to specific T cells. This association supports a significant contribution of the immune system in the pathogenesis of MS.

Table 1. Characteristics of experimental models of multiple sclerosis

Feature	Rodents	Ref.	Nonhuman primates #	Ref.
Predisposition				
Genetic	DR2 (DRB1*1501) DR4 (DRB1*0401) Tg mice	[9,10]	Cj: -DRB*1201; Mm: -DPB1*01	[11,12]
Infection	Virus: SFV, TMEV	[13]	Unknown	
Stress	Lewis rats	[14]	Unknown	
Gender	In some mouse EAE models	[15]	No effect	
Diet	Mouse EAE	[16]	Unknown	
Population	Inbred; F1	[17]	Outbred	
Prevalence	Variable dependent on strain		Mm: MBP 60%; MOG 100% Cj: myelin/MOG 100%; MBP 0%	[12,18]
Clinical				
Induction	Virus models: infection	[13]	EAE: immunization or transfer by CD4+ T cells	[6,19]
	EAE: immunization or transfer with CD4+ T cells	[20]		
Spontaneous	In transgenic animals	[9]	No	
Optic nerve involvement	Virus and EAE models	[21,22]	Cj: MOG/myelin	[6,19]
Variable course of paralysis				[6,19]
Hyperacute	EAE	[23]	Mm: Myelin, MBP, MOG	
Acute	EAE in ABH mice with MBP, MAG	[24,25]	Mm: MOG34-56	
Benign	SFV, EAE		Cj: MBP w/o <i>B. pertussis</i>	
Primary progressive	EAE in C57BK/6	[24]	Cj: MOG	
Relapsing/remitting	MOG, PLP-EAE in ABH mice, TMEV	[26-28]	Cj: Myelin w <i>B. pertussis</i>	
Secondary progressive	MOG, PLP-EAE in ABH mice	[27,28]	Cj: myelin w/o <i>B. pertussis</i>	
Fatigue	Unknown		Unknown	
Depression	EAE in mice	[14]	Unknown	
Physiological				
Visual evoked responses	CREAE, SFV in mice	[29,30]	Unknown	
Blood-brain barrier changes	CREAE, SFV in mice	[31,32]	Cj: myelin/MOG (post Gado MRI)	[33,34]
Diagnosis				
Radiological				
MRI	EAE in rats and mice	[35,36]	Cj: all marmoset models	[33,37]
Laboratory				
CSF oligoclonal bands	TMEV	[38]	Unknown	
CSF pleiocytosis	CREAE in guinea pigs, SFV in mice	[39,40]	Cj and Mm: MBP T-cell transfer	[41,42]
Histopathological				
Inflammation	All models (not chemically induced)		Mm and Cj: all models	[6,19]
Primary	CREAE in mice, guinea pigs, rats, SFV and TMEV in mice	[43-45]	Cj: Myelin and MOG	[11,34,46]
Demyelination		[47]	Mm: myelin and MOG	
Wallarian degeneration	Not known		Cj: Myelin w <i>B. pertussis</i>	[18,48]
Axonal damage	MOG-induced EAE in rats; TMEV in mice	[49,50]	Cj: Myelin w/o <i>B. pertussis</i>	[34]
Remyelination	EAE, TMEV and SFV in mice	[51-53]	Some in myelin EAE in Cj	[34]
PNS involvement	EAE	[54]		
Therapeutic intervention				
Primate specific	Not applicable		Antibodies to CD40 and IL-12	[55••,56,57••]
Adhesion molecules	SFV in mice, EAE	[58-60]		
Immunosuppression	SFV in mice, EAE	[61-63]	Rolipram	[64]
Antigen-specific	EAE in mice, rats	[65,66]	s.c. MBP peptides, i.v. MOG	[19,64]
Cytokine	EAE	[67-70]	IFN β	[19]
IVIg	TMEV, EAE	[71,72]	AntiMOG Fab2	[19]
Altered peptide ligands	EAE	[73,74]		
TcR peptides	EAE	[75]		
Cannabis	EAE in mice	[76]		
Others	Nerve cell growth factor, IFN β (rat EAE)	[67,77]	Nerve growth factor	[78]

Cj, *Callithrix jacchus* or the common marmoset; Mm, *Macaca mulatta* or the rhesus macaque; SFV, Semliki-Forest virus; TMEV, Theiler's murine encephalomyelitis virus; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; PLP, proteolipid protein; MRI, magnetic resonance imaging; CSF, cerebrospinal fluid; PNS, peripheral nervous system; s.c., subcutaneous; i.v., intravenous; CREAE, chronic relapsing EAE.

Animal models of multiple sclerosis

An ideal animal model would reproduce all aspects of MS such as susceptibility, clinical aspects and histopathology (Table 1). This model should also be easy to handle and maintain and, for this reason, laboratory rodents are the most frequently used animals, particularly with the large repertoire of reagents available with which to probe the disease. Furthermore the advances in genetic engineering and the subsequent ease of producing animals with for example dysfunctional genes where genes are deleted (knock-out) or inserted (knock-in) [5]. Transgenic mice are made on the C57BL/6 background – a strain which is susceptible to experimental autoimmune diseases such as experimental allergic encephalomyelitis (EAE) or to virus induced encephalomyelitis following infection with Semliki-Forest (SFV) or Theiler's murine encephalomyelitis virus (TMEV). Such developments have also allowed human genes such as HLA DR2 1501, the class II molecule associated with MS, to be inserted into the mouse for the study of 'MS'-associated immunological responses. In general these mouse strains provide powerful new approaches to study detailed pathological mechanisms, factors and pathways that could provide specific targets for therapeutic strategies. More relevant models of MS are however observed in the nonhuman primates. By virtue of their close immunological and genetic proximity with man, the nonhuman primate models are invaluable for integrating pathology, clinical and magnetic resonance imaging correlates of disease, as well as the design of therapies that are highly species-specific which are impossible in rodents [6].

The pathogenesis of EAE involves complex patterns of cell migration from the peripheral lymphoid organs where they are activated, to the CNS, where they exert their pathogenic effect [7•]. Additional mechanisms may play a role in the perpetuation of the disease, such as the progressive diversification of autoreactive T cells and antibodies, a phenomenon known as epitope spreading [8].

While there are many advantages to using mice, the small size of the brain makes mice less suitable to study lesion formation *in vivo*, such as with nuclear magnetic resonance techniques (magnetic resonance imaging/spectroscopy). Also for studies involving longitudinal sampling of body fluids, bigger species, such as rats or primates, are more suitable.

Rodent models

The concept that the autoimmune reactions in MS are induced by a microbial infection is modelled by experimental infections in genetically susceptible strains of mice, such as intraperitoneal infection of Biozzi ABH or C57BL/6 mice with the avirulent A7(74) strain of SFV

[79], and intracranial infection of SJL/J mice with TMEV [26,47]. Clinical and neuropathological features are reproduced by experimental infection of genetically susceptible strains of mice with viruses such as TMEV or SFV.

Virus-induced encephalomyelitis

TMEV is a naturally occurring neurotropic mouse virus that causes MS-like disease in several strains of mice such as C57BL/6 or SJL mice [80]. Neurovirulent strains of TMEV (GDVII and FA) induce acute fatal encephalitis, while low virulent strains (BeAN and DA) cause persistent infection and demyelination of the CNS, most prevalent in the spinal cord and to a lesser extent in the brainstem, cerebellum and cerebral hemispheres [81]. The exact cause of neurovirulence and persistence of TMEV variants is unknown, but seems to be related to the sialic acid binding intensity of the virus at target cell entry [82]. In CNS areas that are persistently infected with TMEV, chemokines are induced, monocyte chemoattractant protein-1, RANTES and inducible protein-10 [83], which play an important role in the CNS infiltration by peripheral T cells and monocytes. The persistent TMEV infection is not likely to be due to an impaired antiviral immune response as mice susceptible to TMEV-induced encephalomyelitis mount an equally effective cytotoxic immune response to the virus as disease-resistant mice [84•]. The precise effector mechanisms operating in the initiation and perpetuation of demyelination are being elucidated. Demyelination is initiated by CD8+ T cells, which kill virus-infected CNS cells. In addition, myelin reactive CD8+ cells are also induced which may mediate direct Fas–Fas ligand killing of uninfected CNS cells [85•]. The spreading of the antiviral immune reaction to a mainly Th1 cell-mediated anti-myelin autoimmune reaction occurs by molecular mimicry [86]. CD4+ T cells isolated from the spinal cords of TMEV infected SJL mice proliferate and secrete INF γ in response to TMEV epitopes as well as proteolipid protein peptide 139–151 [87]. The main pathogenic role of CD4+ T cells seems to be the local induction of a delayed-type hypersensitivity reaction towards myelin or virus antigens [26]. A recent publication highlights the contribution of immunoglobulin free light chains [88•], a diagnostic marker for MS in the cerebrospinal fluid (CSF) [89], and mast cells, an essential albeit underestimated cell type in the pathogenesis of encephalomyelitis [90,91], to a CD4 cell-mediated delayed-type hypersensitivity reaction.

The progressive demyelination in TMEV-encephalomyelitis is perpetuated by an expanding repertoire of CD4+ autoreactive T cells specific for myelin antigens which are thought to be activated within the CNS by resident antigen-presenting cells (APCs) presenting myelin released from damaged white matter [92•].

SFV-infected Biozzi ABH or C57BL/6 mice develop immune-mediated demyelination of brain and spinal cord white matter and neurological deficit [79,93]. SFV infects brain endothelial cells thereby affecting the blood–brain barrier (BBB) function. Clearance of the virus by a CD8⁺ T-cell mediated immune response is not likely to be the primary cause of demyelination, although CD8⁺ T cells do play a major role in the pathogenesis of SFV [94]. There is clear evidence, however, that demyelination may, in part, be due to the cross-reactivity of anti-SFV T cells and antibodies with structurally similar motifs (mimicry motifs) within myelin antigens, including myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) [95,96].

Blood–brain barrier passage in experimental autoimmune encephalomyelitis

The mechanisms by which autoreactive T and B cells induce MS-like disease in laboratory animals can be investigated in the EAE model. EAE is induced following immunization with myelin and other CNS related antigens together with appropriate adjuvants such as complete Freund's adjuvant. Pathogenic autoreactive CD4⁺ T cells are, however, part of the immune repertoire of rodents and primates which are normally controlled by CD4⁺CD25⁺ regulatory T cells [97]. The tolerant state can be broken by strong activation of the APCs, such as via triggering of CD40 by CD154 ligation or with agonistic antibody and of TLR9 with CpG oligonucleotides [98].

On their way from peripheral lymph nodes, where they are activated, to the CNS, mononuclear cells encounter a major hurdle – the BBB. The BBB is normally impermeable for cells and macromolecules, although activated (CD4⁺) T cells seem to freely transmigrate the BBB [99] by usage of cytoplasmic endoglycosidases [100]. Chemokines regulate T-cell transmigration of the BBB by strengthening their adhesion to inflamed brain venules. The adhesion of encephalitogenic T cells, expressing CCR9 and CXCR3, to the BBB endothelium seems to depend on CCL19 and CCL21, expressed in venules surrounded by inflammatory cells [101]. Using a novel 'intravital microscopy' model and blocking antibodies, the factors that modulate lymphocyte recruitment across inflamed vasculature have been investigated *in vivo*. In EAE tethering and rolling of proteolipid protein 139–151 specific T cells appeared to be mediated by interaction of E- and P-selectins with their ligands, whereas rolling is suggested to be mediated by leukocyte function antigen-1 (LFA-1)/intercellular adhesion molecule-1 (ICAM-1) and α 4 integrin/vascular cell adhesion molecule-1 interaction. LFA-1/ICAM-1 interaction appeared to cause firm arrest of the T cells on endothelium [102]. Such studies have been critical in the development of therapies targeting molecules such as

very late antigen-4 (VLA-4) for the treatment of MS [103]. The BBB is thought to be opened for entry of B cells, macrophages and humoral factors (complement, antibodies) by the action of matrix metalloproteinases [104] and cytokines [105] released by the activated T cells, possibly involving mast cells as well [106]. The CNS immigration of monocytes/macrophages is not random, but directed by locally produced chemokines [107].

Central nervous system immigration

CNS infiltrating T cells are thought to acquire the capacity to penetrate the CNS parenchyma during cognate interaction with APCs at the BBB, presenting their specific antigen. C57BL/6 mice with a genetical MHC class II transactivator deficiency are resistant to EAE induced either by active immunization or by transfer of encephalitogenic T cells from MOG35–55-immunized wild-type mice. These mice have disturbed antigen presentation capacity by defective MHC class II, invariant chain and H-2M (DM) expression. Intriguingly, DM-deficient mice can generate a normal Th1 response to MOG35–55, which induce EAE by transfer into wild-type mice, but not in DM-deficient mice [108]. This strongly suggests that processing and presentation of myelin antigens by resident APCs within the CNS is essential for EAE induction. The intraparenchymal interaction of infiltrated T cells with local APCs induces a pathophysiological cascade of reactions involving soluble factors, including antibodies, complement, cytokines and matrix metalloproteinases, that trigger white matter inflammation and demyelination.

Induction of lesions and neurological deficit

B-cell deficient mice, such as μ MT on C57BL/10 or DBA/1 background, develop less severe EAE after immunization with MOG (1–125) in complete Freund's adjuvant. While demyelination is significantly reduced compared with wild-type mice, the influx of inflammatory cells is hardly affected [109]. A second study addressing the role of B cells in EAE showed that transfer of activated B cells or serum from MOG (1–125) immunized mice restores the capacity of B-cell deficient mice to develop EAE. Lipopolysaccharide-activated B cells or immune sera from mice immunized with irrelevant antigens had no effect. It is particularly noteworthy that serum from mice immunized with an immunodominant (MOG 35–55) peptide had no effect [109]. While MOG specific antibodies are well known to exacerbate clinical disease and to induce demyelination in rats and mice, immunological responses to galactocerebroside are also important both for exacerbating clinical disease and pathological effects [110], while anti-MBP antibody has been shown to reduced disease severity [24]. A highly unexpected finding is

that B cells are also capable of controlling the activity of Th1 cells, namely by antigen-induced production of IL-10 [111**]. Collectively, these data show that the impact of B cells on EAE goes further than only the facilitation of demyelination, but also includes immunoregulation.

While the immune mechanisms that cause CNS inflammation and demyelination have been investigated in detail in the past few decades, the cause of the neurological deficit in MS and EAE is still poorly understood. It is clear from animal models that CNS inflammation and demyelination do not fully account for the irreversible neurological deficit in advanced MS. In a seminal paper, Trapp *et al.* [112] have clearly shown significant axonal pathology in MS lesions that cannot be ignored as a possible cause of neurological dysfunction. The finding that significant axonal pathology occurs also in EAE opens the possibility of developing neuroprotective therapies [49,113].

In conclusion, rodent models of MS have been critical for the study of the basic mechanisms of recruitment of inflammation within the CNS as well as mechanisms leading to myelin and axonal damage. These studies have formed the basis for the development of therapeutic strategies in more appropriate models of MS, namely nonhuman primates.

Experimental autoimmune encephalomyelitis in nonhuman primates

Based on the increased insight into the pathogenic mechanisms operating in rodent EAE models, intervention strategies are being developed which are of potential benefit to MS [114]. Some examples are antibodies directed against effector cells or molecules, peptides that block antigen receptors on T or B cells or APC molecules that cause deviation of a proinflammatory Th1 reaction to a protective Th2 reaction, or viral vectors that express therapeutic molecules. An interesting new strategy is to use anti-MOG IgG-Fab fragments generated from a combinatorial library for the inhibition of autoantibody-mediated demyelination [115].

In many cases, therapies based on biological reagents are highly species-specific. This implies that reagents developed for treatment of humans should be tested in nonhuman primates. Of the wide variety of immunosuppressive/modulatory therapies that were tested in MS after proven effectivity in EAE models, only very few have shown beneficial effect in patients [116]. In our view, these failures illustrate the need for a valid preclinical model that is more closely related to the human disease. The common marmoset, a small Neotropical primate with significant genetic and immunological similarity to humans [117–119], provides an

excellent model that approximates chronic MS by the clinical and neuropathological presentation [120].

IL-12 is involved in the induction of a proinflammatory immune reaction by the polarization of antigen-specific T cells into the Th1 direction. IL-12 is produced at sites where T cells and APCs interact by the interaction of CD40 with its ligand CD154 [55**,121]. IL-12 seems to be of critical importance in EAE due to its central role in the regulation of tolerance and autoimmunity [122]. Using immunohistochemistry, similar staining patterns for CD40 and CD154 have been found in lesions of MS patients as in EAE-affected mice or common marmosets [123,124]. The relevance of CD40–CD154 interaction seems particularly essential for the induction of proinflammatory T cells in EAE when CD28 is absent [125]. Lesions in EAE-affected marmosets also stain positively with anti-human IL-12p40 antibody. Antibodies directed to CD40 or IL-12p40 have a significant inhibitory effect on lesion formation and the expression of neurological deficit in marmosets, confirming the important role of the CD40–IL-12 pathway in the disease pathogenesis [55**,56,57**]. At this stage we cannot formally exclude that the effect of anti-IL-12p40 antibody is actually exerted via IL-23, a heterodimer of a IL-12p40 and p19 subunit with overlapping activity with IL-12 [126]. EAE experiments in IL-12p35 or IL-12p40 deficient mice support this possibility. The reported inhibitory effect on EAE in SJL mice of curcumin, a natural plant phenol that inhibits IL-12, suggests a role for this cytokine in the disease [127]. While p35–/– and p40–/– mice both fail to produce the functional IL-12p70 heterodimer, however, only mice deficient in the p40 subunit which is shared by IL-12 and IL-23 are resistant to EAE [128*].

An interesting aspect of anti-CD40 antibody treatment in rhMOG-immunized marmosets is that the initial T- and B-cell autoreactivity does not seem to be affected, but that the progressive diversification of the autoimmune reaction is suppressed [56]. The pathogenic function of epitope spreading has long been debated. Two recent papers, however, support our hypothesis that disease and epitope spreading are connected. First, the observation that superantigenic stimulation (staphylococcal enterotoxin) of PL/J mice, in remission from MBP-induced EAE, induces exacerbation of clinical signs and epitope spreading [129]; second, the finding of regulatory T cells specific for spreading epitopes that can reverse ongoing disease [8].

A recent publication points to a mechanism underlying epitope diversification [130**]. Immunohistochemical analysis of the brain draining cervical lymph nodes of EAE-affected marmosets and rhesus monkeys shows localization of myelin loaded cells with macrophage/DC-specific markers in close connection with naïve T and B

cells. As such cells are absent in the cervical lymph nodes of marmosets immunized with ovalbumin or rhesus monkeys immunized with joint cartilage specific type II collagen, they may probably originate from macrophages which have phagocytosed myelin in the lesions. Dendritic cells pulsed with peptides from myelin proteins are potent inducers of autoreactive T cells and disease [131]. We hypothesize, therefore, that the myelin-loaded APCs present in the cervical lymph nodes activate the progressively broadening repertoire of anti-myelin T and B cells that can be detected in chronic EAE [11].

Conclusion

In this review we have discussed the use of animal models for MS. While many aspects of MS are still unclear, the use of an experimental model is critical to determine the mechanisms of CNS damage that may be operating in MS, as well as to provide the tools to evaluate the possible strategies to control the disease. The past years have shown an increasing effort to translate new insights obtained in animal models into new therapeutic principles for MS. Areas of particular importance for the coming decade include not only clear differentiation of the different types of MS, but also the analysis of genes and proteins that are differentially expressed in the different types of MS lesions compared with normal white matter [132]. While many animal models are available, the use of relevant animal models that mimic either the different forms of MS or the spectrum of MS are critical to examine those factors, for example genes or proteins, that are of pathogenic relevance and can be used as targets for therapy.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

- 1 Compston A, Coles A. Multiple sclerosis. *Lancet* 2002; 359:1221–1231.
- 2 Lucchinetti C, Bruck W, Parisi J, *et al.* Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol* 2000; 47:707–717.
- 3 Pette M, Fujita K, Wilkinson D, *et al.* Myelin autoreactivity in multiple sclerosis: recognition of myelin basic protein in the context of HLA-DR2 products by T lymphocytes of multiple-sclerosis patients and healthy donors. *Proc Natl Acad Sci U S A* 1990; 87:7968–7972.
- 4 Sawcer S, Maranian M, Setakis E, *et al.* A whole genome screen for linkage disequilibrium in multiple sclerosis confirms disease associations with regions previously linked to susceptibility. *Brain* 2002; 125:1337–1347.
- 5 Owens T, Wekerle H, Antel J. Genetic models for CNS inflammation. *Nat Med* 2001; 7:161–166.
- 6 Brok HP, Bauer J, Jonker M, *et al.* Non-human primate models of multiple sclerosis. *Immunol Rev* 2001; 183:173–185.
- 7 Flugel A, Berkowicz T, Ritter T, *et al.* Migratory activity and functional changes of green fluorescent effector cells before and during experimental autoimmune encephalomyelitis. *Immunity* 2001; 14:547–560.
- In this seminal publication the homing patterns of adoptively transferred GFP transfected T-cell clones specific for MBP or OVA were investigated. The investigators show that both clones migrate from parathymic lymph nodes via the blood to the spleen where they stay latent for several days. Just prior to EAE onset MBP-specific T cells migrate to the CNS, while OVA-specific T cells are drained to lymph nodes.
- 8 Wildbaum G, Netzer N, Karin N. Tr1 cell-dependent active tolerance blunts the pathogenic effects of determinant spreading. *J Clin Invest* 2002; 110:701–710.
- 9 Boyton RJ, Altmann DM. Transgenic models of autoimmune disease. *Clin Exp Immunol* 2002; 127:4–11.
- 10 Forsthuber TG, Shive CL, Wienhold W, *et al.* T cell epitopes of human myelin oligodendrocyte glycoprotein identified in HLA-DR4 (DRB1*0401) transgenic mice are encephalitogenic and are presented by human B cells. *J Immunol* 2001; 167:7119–7125.
- 11 Brok HP, Uccelli A, Kerlero De Rosbo N, *et al.* Myelin/oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis in common marmosets: the encephalitogenic T cell epitope pMOG24-36 is presented by a monomorphic MHC class II molecule. *J Immunol* 2000; 165:1093–1101.
- 12 Sliemers BL, Hall M, 't Hart B, *et al.* Identification of an Mhc-DBP1 allele involved in susceptibility to experimental autoimmune encephalomyelitis in rhesus macaques. *Int Immunol* 1995; 7:1671–1679.
- 13 Atkins GJ, McQuaid S, Morris-Downes MM, *et al.* Transient virus infection and multiple sclerosis. *Rev Med Virol* 2000; 10:291–303.
- 14 Stephan M, Straub RH, Breivik T, *et al.* Postnatal maternal deprivation aggravates experimental autoimmune encephalomyelitis in adult Lewis rats: reversal by chronic imipramine treatment. *Int J Dev Neurosci* 2002; 20:125–132.
- 15 Voskuhl RR, Palaszynski K. Sex hormones in experimental autoimmune encephalomyelitis: implications for multiple sclerosis. *Neuroscientist* 2001; 7:258–270.
- 16 Harbige LS, Jones R, Jenkins R, *et al.* Nutritional management in multiple sclerosis with reference to experimental models. *Ups J Med Sci Suppl* 1990; 48:189–207.
- 17 Sobel RA. Genetic and epigenetic influence on EAE phenotypes induced with different encephalitogenic peptides. *J Neuroimmunol* 2000; 108:45–52.
- 18 Kerlero de Rosbo N, Brok HP, Bauer J, *et al.* Rhesus monkeys are highly susceptible to experimental autoimmune encephalomyelitis induced by myelin oligodendrocyte glycoprotein: characterisation of immunodominant T- and B-cell epitopes. *J Neuroimmunol* 2000; 110:83–96.
- 19 Genain CP, Hauser SL. Experimental allergic encephalomyelitis in the New World monkey *Callithrix jacchus*. *Immunol Rev* 2001; 183:159–172.
- 20 Pettinelli CB, McFarlin DE. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1+ 2-T lymphocytes. *J Immunol* 1981; 127:1420–1423.
- 21 Butt AM, Tutton MG, Kirvell SL, *et al.* Morphology of oligodendrocytes during demyelination in optic nerves of mice infected with Semliki Forest virus. *Neuropathol Appl Neurobiol* 1996; 22:540–547.
- 22 O'Neill JK, Baker D, Morris MM, *et al.* Optic neuritis in chronic relapsing experimental allergic encephalomyelitis in Biozzi ABH mice: demyelination and fast axonal transport changes in disease. *J Neuroimmunol* 1998; 82:210–218.
- 23 MacMicking JD, Willenborg DO, Weidemann MJ, *et al.* Elevated secretion of reactive nitrogen and oxygen intermediates by inflammatory leukocytes in hyperacute experimental autoimmune encephalomyelitis: enhancement by the soluble products of encephalitogenic T cells. *J Exp Med* 1992; 176:303–307.
- 24 Morris-Downes MM, McCormack K, Baker D, *et al.* Encephalitogenic and immunogenic potential of myelin-associated glycoprotein (MAG), oligodendrocyte-specific glycoprotein (OSP) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) in ABH and SJL mice. *J Neuroimmunol* 2002; 122:20–33.
- 25 Amor S, O'Neill JK, Morris MM, *et al.* Encephalitogenic epitopes of myelin basic protein, proteolipid protein, myelin oligodendrocyte glycoprotein for experimental allergic encephalomyelitis induction in Biozzi ABH (H-2Ag7) mice share an amino acid motif. *J Immunol* 1996; 156:3000–3008.
- 26 Dal Canto MC, Kim BS, Miller SD, *et al.* Theiler's murine encephalomyelitis virus (TMEV)-induced demyelination: a model for human multiple sclerosis. *Methods* 1996; 10:453–461.

- 27 Amor S, Groome N, Linington C, *et al.* Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. *J Immunol* 1994; 153:4349–4356.
 - 28 Amor S, Baker D, Groome N, *et al.* Identification of a major encephalitogenic epitope of proteolipid protein (residues 56–70) for the induction of experimental allergic encephalomyelitis in Biozzi AB/H and nonobese diabetic mice. *J Immunol* 1993; 150:5666–5672.
 - 29 Tremain KE, Ikeda H. Physiological deficits in the visual system of mice infected with Semliki Forest virus and their correlation with those seen in patients with demyelinating disease. *Brain* 1983; 106 (Pt 4):879–895.
 - 30 Bilboul N, Kaitz M, Feinsod M, *et al.* Visual evoked potentials in experimental allergic encephalomyelitis. *J Neurol Sci* 1983; 60:105–115.
 - 31 Pan W, Banks WA, Kennedy MK, *et al.* Differential permeability of the BBB in acute EAE: enhanced transport of TNT- α . *Am J Physiol Endocrinol Metab* 1996; 271:E636–E642.
 - 32 Eralinna JP, Soilu-Hanninen M, Roytta M, *et al.* Blood–brain barrier breakdown and increased intercellular adhesion molecule (ICAM-1/CD54) expression after Semliki Forest (A7) virus infection facilitates the development of experimental allergic encephalomyelitis. *J Neuroimmunol* 1996; 66:103–114.
 - 33 Jordan EK, McFarland HI, Lewis BK, *et al.* Serial MR imaging of experimental autoimmune encephalomyelitis induced by human white matter or by chimeric myelin-basic and proteolipid protein in the common marmoset. *AJNR Am J Neuroradiol* 1999; 20:965–976.
 - 34 't Hart BA, Bauer J, Muller HJ, *et al.* Histopathological characterization of magnetic resonance imaging-detectable brain white matter lesions in a primate model of multiple sclerosis: a correlative study in the experimental autoimmune encephalomyelitis model in common marmosets (*Callithrix jacchus*). *Am J Pathol* 1998; 153:649–663.
 - 35 Karlik SJ, Munoz D, St Louis J, *et al.* Correlation between MRI and clinicopathological manifestations in Lewis rats protected from experimental allergic encephalomyelitis by acylated synthetic peptide of myelin basic protein. *Magn Reson Imaging* 1999; 17:731–737.
 - 36 Xu S, Jordan EK, Brocke S, *et al.* Study of relapsing remitting experimental allergic encephalomyelitis SJL mouse model using MION-46L enhanced in vivo MRI: early histopathological correlation. *J Neurosci Res* 1998; 52:549–558.
 - 37 Blezer ELA, Brok HPM, Nicolay K, *et al.* Quantifying the progressive inflammatory demyelination of brain white matter in MOG-immunized common marmosets with magnetic resonance imaging (in press).
 - 38 Roos RP, Nalefski EA, Nitayaphan S, *et al.* An isoelectric focusing overlay study of the humoral immune response in Theiler's virus demyelinating disease. *J Neuroimmunol* 1987; 13:305–314.
 - 39 Suckling AJ, Baron PW, Wilson NR, *et al.* Activated T-cells and macrophages in the cerebrospinal fluid and the spinal meningeal exudate in chronic relapsing experimental allergic encephalomyelitis. *J Neurol Sci* 1987; 78:241–252.
 - 40 Parsons LM, Webb HE. Virus titres and persistently raised white cell counts in cerebrospinal fluid in mice after peripheral infection with demyelinating Semliki Forest virus. *Neuropathol Appl Neurobiol* 1982; 8:395–401.
 - 41 Genain CP, Lee-Parritz D, Nguyen MH, *et al.* In healthy primates, circulating autoreactive T cells mediate autoimmune disease. *J Clin Invest* 1994; 94:1339–1345.
 - 42 Meinel E, Hoch RM, Dornmair K, *et al.* Encephalitogenic potential of myelin basic protein-specific T cells isolated from normal rhesus macaques. *Am J Pathol* 1997; 150:445–453.
 - 43 Raine CS, Barnett LB, Brown A, *et al.* Neuropathology of experimental allergic encephalomyelitis in inbred strains of mice. *Lab Invest* 1980; 43:150–157.
 - 44 Pender MP, Stanley GP, Yoong G, *et al.* The neuropathology of chronic relapsing experimental allergic encephalomyelitis induced in the Lewis rat by inoculation with whole spinal cord and treatment with cyclosporin A. *Acta Neuropathol (Berl)* 1990; 80:172–183.
 - 45 dal Canto MC, Lipton HL. A new model of persistent viral infection with primary demyelination. *Neurol Neurocir Psychiatr* 1977; 18:455–467.
 - 46 Massaccesi L, Genain CP, Lee-Parritz D, *et al.* Active and passively induced experimental autoimmune encephalomyelitis in common marmosets: a new model for multiple sclerosis. *Ann Neurol* 1995; 37:519–530.
 - 47 Amor S, Scallan MF, Morris MM, *et al.* Role of immune responses in protection and pathogenesis during Semliki Forest virus encephalitis. *J Gen Virol* 1996; 77 (Pt 2):281–291.
 - 48 Poliani PL, Brok H, Furlan R, *et al.* Delivery to the central nervous system of a nonreplicative herpes simplex type 1 vector engineered with the interleukin 4 gene protects rhesus monkeys from hyperacute autoimmune encephalomyelitis. *Hum Gene Ther* 2001; 12:905–920.
 - 49 Kornek B, Storch MK, Weissert R, *et al.* Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. *Am J Pathol* 2000; 157:267–276.
 - 50 McGavern DB, Murray PD, Rivera-Quinones C, *et al.* Axonal loss results in spinal cord atrophy, electrophysiological abnormalities and neurological deficits following demyelination in a chronic inflammatory model of multiple sclerosis. *Brain* 2000; 123 (Pt 3):519–531.
 - 51 Brown A, McFarlin DE, Raine CS. Chronologic neuropathology of relapsing experimental allergic encephalomyelitis in the mouse. *Lab Invest* 1982; 46:171–185.
 - 52 McGavern DB, Murray PD, Rodriguez M. Quantitation of spinal cord demyelination, remyelination, atrophy, and axonal loss in a model of progressive neurologic injury. *J Neurosci Res* 1999; 58:492–504.
 - 53 Kelly WR, Blakemore WF, Jagelman S, *et al.* Demyelination induced in mice by avirulent Semliki Forest virus: II. An ultrastructural study of focal demyelination in the brain. *Neuropathol Appl Neurobiol* 1982; 8:43–53.
 - 54 Berger T, Weerth S, Kojima K, *et al.* Experimental autoimmune encephalomyelitis: the antigen specificity of T lymphocytes determines the topography of lesions in the central and peripheral nervous system. *Lab Invest* 1997; 76:355–364.
 - 55 Laman JD, 't Hart BA, Brok H, *et al.* Protection of marmoset monkeys against EAE by treatment with a murine antibody blocking CD40 (mu5D12). *Eur J Immunol* 2002; 32:2218–2228.
- The authors demonstrate that antibody-mediated blocking of CD40 is an effective target to protect common marmosets immunized with MS patient myelin to MS-like disease. Evidence is provided that the antibody does not only work in secondary lymphoid organs, but also inside the CNS white matter lesions.
- 56 Boon L, Brok HP, Bauer J, *et al.* Prevention of experimental autoimmune encephalomyelitis in the common marmoset (*Callithrix jacchus*) using a chimeric antagonist monoclonal antibody against human CD40 is associated with altered B cell responses. *J Immunol* 2001; 167:2942–2949.
 - 57 Brok HP, Van Meurs M, Blezer E, *et al.* Prevention of experimental autoimmune encephalomyelitis in common marmosets using an anti-IL-12p40 monoclonal antibody. *J Immunol* 2002; 169:6554–6563.
- In this publication we demonstrate the strong beneficial effect of an antibody directed to IL-12p40 in a valid preclinical model of MS, myelin-induced EAE in the New World primate species common marmosets. As the antibody is virtually nonimmunogenic and signs of toxicity are lacking these findings warrant clinical evaluation of the antibody for treatment of MS.
- 58 Soilu-Hanninen M, Roytta M, Salmi A, *et al.* Therapy with antibody against leukocyte integrin VLA-4 (CD49d) is effective and safe in virus-facilitated experimental allergic encephalomyelitis. *J Neuroimmunol* 1997; 72:95–105.
 - 59 Smith JP, Morris-Downes M, Brennan FR, *et al.* A role for alpha4-integrin in the pathology following Semliki Forest virus infection. *J Neuroimmunol* 2000; 106:60–68.
 - 60 van der Laan LJ, van der Goes A, Wauben MH, *et al.* Beneficial effect of modified peptide inhibitor of alpha4 integrins on experimental allergic encephalomyelitis in Lewis rats. *J Neurosci Res* 2002; 67:191–199.
 - 61 Amor S, Webb HE. The effect of cycloleucine on SFV A7(74) infection in mice. *Br J Exp Pathol* 1987; 68:225–235.
 - 62 Fazakerley JK, Webb HE. Semliki Forest virus induced, immune mediated demyelination: the effect of irradiation. *Br J Exp Pathol* 1987; 68:101–113.
 - 63 Levine S, Saltzman A. Regional suppression, therapy after onset and prevention of relapses in experimental allergic encephalomyelitis by mitoxantrone. *J Neuroimmunol* 1986; 13:175–181.
 - 64 Genain CP, Nguyen MH, Letvin NL, *et al.* Antibody facilitation of multiple sclerosis-like lesions in a nonhuman primate. *J Clin Invest* 1995; 96:2966–2974.
 - 65 Jewell SD, Gienapp IE, Cox KL, *et al.* Oral tolerance as therapy for experimental autoimmune encephalomyelitis and multiple sclerosis: demonstration of T cell anergy. *Immunol Cell Biol* 1998; 76:74–82.
 - 66 Maron R, Slavov AJ, Hoffmann E, *et al.* Oral tolerance to copolymer 1 in myelin basic protein (MBP) TCR transgenic mice: cross-reactivity with MBP-specific TCR and differential induction of anti-inflammatory cytokines. *Int Immunol* 2002; 14:131–138.
 - 67 Floris S, Ruuls SR, Wierinckx A, *et al.* Interferon-beta directly influences monocyte infiltration into the central nervous system. *J Neuroimmunol* 2002; 127:69–79.

- 68 Bright JJ, Sriram S. Immunotherapy of inflammatory demyelinating diseases of the central nervous system. *Immunol Res* 2001; 23:245–252.
- 69 Chambers CA, Kuhns MS, Egen JG, *et al.* CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annu Rev Immunol* 2001; 19:565–594.
- 70 Selmaj KW. Tumour necrosis factor and anti-tumour necrosis factor approach to inflammatory demyelinating diseases of the central nervous system. *Ann Rheum Dis* 2000; 59 (Suppl 1):i94–i102.
- 71 Noseworthy JH, O'Brien PC, van Engelen BG, *et al.* Intravenous immunoglobulin therapy in multiple sclerosis: progress from remyelination in the Theiler's virus model to a randomised, double-blind, placebo-controlled clinical trial. *J Neurol Neurosurg Psychiatry* 1994; 57 (Suppl):11–14.
- 72 Achiron A, Gilad R, Margalit R, *et al.* Intravenous gammaglobulin treatment in multiple sclerosis and experimental autoimmune encephalomyelitis: delineation of usage and mode of action. *J Neurol Neurosurg Psychiatry* 1994; 57 (Suppl):57–61.
- 73 Young DA, Lowe LD, Booth SS, *et al.* IL-4, IL-10, IL-13, and TGF-beta from an altered peptide ligand-specific Th2 cell clone down-regulate adoptive transfer of experimental autoimmune encephalomyelitis. *J Immunol* 2000; 164:3563–3572.
- 74 Nicholson LB, Greer JM, Sobel RA, *et al.* An altered peptide ligand mediates immune deviation and prevents autoimmune encephalomyelitis. *Immunity* 1995; 3:397–405.
- 75 Offner H, Jacobs R, Bebo BF Jr, *et al.* Treatments targeting the T cell receptor (TCR): effects of TCR peptide-specific T cells on activation, migration, and encephalitogenicity of myelin basic protein-specific T cells. *Springer Semin Immunopathol* 1999; 21:77–90.
- 76 Baker D, Pryce G, Croxford JL, *et al.* Cannabinoids control spasticity and tremor in a multiple sclerosis model. *Nature* 2000; 404:84–87.
- 77 Linker RA, Maurer M, Gaupp S, *et al.* CNTF is a major protective factor in demyelinating CNS disease: a neurotrophic cytokine as modulator in neuroinflammation. *Nat Med* 2002; 8:620–624.
- 78 Villoslada P, Hauser SL, Bartke I, *et al.* Human nerve growth factor protects common marmosets against autoimmune encephalomyelitis by switching the balance of T helper cell type 1 and 2 cytokines within the central nervous system. *J Exp Med* 2000; 191:1799–1806.
- 79 Fazakerley JK, Amor S, Webb HE. Reconstitution of Semliki forest virus infected mice, induces immune mediated pathological changes in the CNS. *Clin Exp Immunol* 1983; 52:115–120.
- 80 Miller SD, Olson JK, Croxford JL. Multiple pathways to induction of virus-induced autoimmune demyelination: lessons from Theiler's virus infection. *J Autoimmun* 2001; 16:219–227.
- 81 Trotter M, Schlitt BP, Lipton HL. Enhanced detection of Theiler's virus RNA copy equivalents in the mouse central nervous system by real-time RT-PCR. *J Virol Meth* 2002; 103:89–99.
- 82 Jnaoui K, Minet M, Michiels T. Mutations that affect the tropism of DA and GDVII strains of Theiler's virus in vitro influence sialic acid binding and pathogenicity. *J Virol* 2002; 76:8138–8147.
- 83 Ransohoff RM, Wei T, Pavelko KD, *et al.* Chemokine expression in the central nervous system of mice with a viral disease resembling multiple sclerosis: roles of CD4+ and CD8+ T cells and viral persistence. *J Virol* 2002; 76:2217–2224.
- 84 Kang BS, Lyman MA, Kim BS. The majority of infiltrating CD8+ T cells in the central nervous system of susceptible SJL/J mice infected with Theiler's virus are virus specific and fully functional. *J Virol* 2002; 76:6577–6585.
- The authors use pathogenic and nonpathogenic strains of the virus to unravel the pathogenic role of CD8+ T cells. It is shown that the outcome of the disease is related to the level of CD8+ T cells and their avidity for viral antigens.
- 85 Tsunoda I, Kuang LQ, Fujinami RS. Induction of autoreactive CD8(+) cytotoxic T cells during Theiler's murine encephalomyelitis virus infection: implications for autoimmunity. *J Virol* 2002; 76:12834–12844.
- The neurotropic virus TMEV induces spinal cord demyelination in susceptible mouse strains by a combination of CD8 cell-mediated antiviral and CD4 cell-mediated anti-self immune responses. The novel finding in this publication is that infection with TMEV induces CD8+ T cells which kill both TMEV-infected and uninfected spleen cells. This finding strongly suggests that CD8+ T cells responding to self antigens are formed. The killing mechanism of these cells (by ligation of Fas with Fas ligand) differs from the killing mechanism of conventional cytotoxic T cells (by cytotoxic granules).
- 86 Olson JK, Eagar TN, Miller SD. Functional activation of myelin-specific T cells by virus-induced molecular mimicry. *J Immunol* 2002; 169:2719–2726.
- 87 Neville KL, Padilla J, Miller SD. Myelin-specific tolerance attenuates the progression of a virus-induced demyelinating disease: implications for the treatment of MS. *J Neuroimmunol* 2002; 123:18–29.
- 88 Redegeld FA, van der Heijden MW, Kool M, *et al.* Immunoglobulin-free light chains elicit immediate hypersensitivity-like responses. *Nat Med* 2002; 8:694–701.
- This publication documents a novel activation mechanism of mast cells by Ig free light chains (IgLC) in a mouse contact allergy model. The specific inhibitor of IgLC binding to its receptor on mast cells should make it possible to determine the elusive pathogenic role of IgLC and mast cells in the pathogenesis of MS.
- 89 Jenkins MA, Cheng L, Ratnaik S. Multiple sclerosis: use of light-chain typing to assist diagnosis. *Ann Clin Biochem* 2001; 38:235–241.
- 90 Secor VH, Secor WE, Gutekunst CA, *et al.* Mast cells are essential for early onset and severe disease in a murine model of multiple sclerosis. *J Exp Med* 2000; 191:813–822.
- 91 Brown M, Tanzola M, Robbie-Ryan M. Mechanisms underlying mast cell influence on EAE disease course. *Mol Immunol* 2002; 38:1373–1378.
- 92 Tompkins SM, Padilla J, Dal Canto MC, *et al.* De novo central nervous system processing of myelin antigen is required for the initiation of experimental autoimmune encephalomyelitis. *J Immunol* 2002; 168:4173–4183.
- According to a central dogma in MS/EAE, activated CD4+ T cells can freely enter perivascular space within the CNS, but that lesion induction cognate interaction with resident APCs presenting their specific antigen is needed. In this technically highly elegant publication using mice with genetic deficiencies in antigen presentation it is shown that this requires processing and presentation of myelin antigens.
- 93 Morris MM, Dyson H, Baker D, *et al.* Characterization of the cellular and cytokine response in the central nervous system following Semliki Forest virus infection. *J Neuroimmunol* 1997; 74:185–197.
- 94 Subak-Sharpe I, Dyson H, Fazakerley J. In vivo depletion of CD8+ T cells prevents lesions of demyelination in Semliki Forest virus infection. *J Virol* 1993; 67:7629–7633.
- 95 Mokhtarian F, Zhang Z, Shi Y, *et al.* Molecular mimicry between a viral peptide and a myelin oligodendrocyte glycoprotein peptide induces autoimmune demyelinating disease in mice. *J Neuroimmunol* 1999; 95:43–54.
- 96 Smith-Norowitz TA, Sobel RA, Mokhtarian F. B cells and antibodies in the pathogenesis of myelin injury in Semliki Forest Virus encephalomyelitis. *Cell Immunol* 2000; 200:27–35.
- 97 Kohm AP, Carpentier PA, Anger HA, *et al.* Cutting edge: CD4(+)CD25(+) regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J Immunol* 2002; 169:4712–4716.
- 98 Ichikawa HT, Williams LP, Segal BM. Activation of APCs through CD40 or toll-like receptor 9 overcomes tolerance and precipitates autoimmune disease. *J Immunol* 2002; 169:2781–2787.
- 99 Hickey WF, Hsu BL, Kimura H. T-lymphocyte entry into the central nervous system. *J Neurosci Res* 1991; 28:254–260.
- 100 Naparstek Y, Cohen IR, Fuks Z, *et al.* Activated T lymphocytes produce a matrix-degrading heparan sulphate endoglycosidase. *Nature* 1984; 310:241–244.
- 101 Alt C, Laschinger M, Engelhardt B. Functional expression of the lymphoid chemokines CCL19 (ELC) and CCL 21 (SLC) at the blood-brain barrier suggests their involvement in G-protein-dependent lymphocyte recruitment into the central nervous system during experimental autoimmune encephalomyelitis. *Eur J Immunol* 2002; 32:2133–2144.
- 102 Piccio L, Rossi B, Scarpini E, *et al.* Molecular mechanisms involved in lymphocyte recruitment in inflamed brain microvessels: critical roles for P-selectin glycoprotein ligand-1 and heterotrimeric G(i)-linked receptors. *J Immunol* 2002; 168:1940–1949.
- 103 Miller DH, Khan OA, Sheremata WA, *et al.* A controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med* 2003; 348:15–23.
- 104 Brundula V, Rewcastle NB, Metz LM, *et al.* Targeting leukocyte MMPs and transmigration: minocycline as a potential therapy for multiple sclerosis. *Brain* 2002; 125:1297–1308.
- 105 Morganti-Kossmann MC, Rancan M, Stahel PF, *et al.* Inflammatory response in acute traumatic brain injury: a double-edged sword. *Curr Opin Crit Care* 2002; 8:101–105.
- 106 Esposito P, Chandler N, Kandere K, *et al.* Corticotropin-releasing hormone and brain mast cells regulate blood-brain-barrier permeability induced by acute stress. *J Pharmacol Exp Ther* 2002; 303:1061–1066.
- 107 Izikson L, Klein RS, Luster AD, *et al.* Targeting monocyte recruitment in CNS autoimmune disease. *Clin Immunol* 2002; 103:125–131.
- 108 Tompkins SM, Fuller KG, Miller SD. Theiler's virus-mediated autoimmunity: local presentation of CNS antigens and epitope spreading. *Ann N Y Acad Sci* 2002; 958:26–38.

- 109 Svensson L, Abdul-Majid KB, Bauer J, *et al.* A comparative analysis of B cell-mediated myelin oligodendrocyte glycoprotein-experimental autoimmune encephalomyelitis pathogenesis in B cell-deficient mice reveals an effect on demyelination. *Eur J Immunol* 2002; 32:1939–1946.
- 110 Raine CS, Traugott U. The pathogenesis and therapy of multiple sclerosis is based upon the requirement of a combination of myelin antigens for autoimmune demyelination. *J Neuroimmunol* 1982; 2:83–91.
- 111 Fillatreau S, Sweeney CH, McGeachy MJ, *et al.* B cells regulate autoimmunity •• by provision of IL-10. *Nat Immunol* 2002; 3:944–950.
The main pathogenic role of B cells in MS/EAE is the production of the autoantibodies that mediate demyelination via the opsonization of the myelin sheaths. A regulatory role of this cell type is not yet known. Evidence for such a function is given in this publication, documenting that autoantigen-reactive T cells in EAE produce the antiinflammatory cytokine IL-10.
- 112 Trapp BD, Peterson J, Ransohoff RM, *et al.* Axonal transection in the lesions of multiple sclerosis. *N Engl J Med* 1998; 338:278–285.
- 113 Wujek JR, Bjartmar C, Richer E, *et al.* Axon loss in the spinal cord determines permanent neurological disability in an animal model of multiple sclerosis. *J Neuropathol Exp Neurol* 2002; 61:23–32.
- 114 Hohlfeld R. Biotechnological agents for the immunotherapy of multiple sclerosis: principles, problems and perspectives. *Brain* 1997; 120 (Pt 5):865–916.
- 115 von Budingen HC, Hauser SL, Fuhrmann A, *et al.* Molecular characterization of antibody specificities against myelin/oligodendrocyte glycoprotein in autoimmune demyelination. *Proc Natl Acad Sci U S A* 2002; 99:8207–8212.
- 116 Pender MP, Wolfe NP. Prevention of autoimmune attack and disease progression in multiple sclerosis: current therapies and future prospects. *Intern Med J* 2002; 32:554–563.
- 117 Brok HP, Hornby RJ, Griffiths GD, *et al.* An extensive monoclonal antibody panel for the phenotyping of leukocyte subsets in the common marmoset and the cotton-top tamarin. *Cytometry* 2001; 45:294–303.
- 118 Bontrop RE, Otting N, de Groot NG, *et al.* Major histocompatibility complex class II polymorphisms in primates. *Immunol Rev* 1999; 167:339–350.
- 119 Uccelli A, Oksenberg JR, Jeong MC, *et al.* Characterization of the TCRB chain repertoire in the New World monkey *Callithrix jacchus*. *J Immunol* 1997; 158:1201–1207.
- 120 't Hart BA, van Meurs M, Brok HP, *et al.* A new primate model for multiple sclerosis in the common marmoset. *Immunol Today* 2000; 21:290–297.
- 121 van Kooten C, Banchereau J. Functional role of CD40 and its ligand. *Int Arch Allergy Immunol* 1997; 113:393–399.
- 122 Zhang GX, Xu H, Kishi M, *et al.* The role of IL-12 in the induction of intravenous tolerance in experimental autoimmune encephalomyelitis. *J Immunol* 2002; 168:2501–2507.
- 123 Gerritse K, Laman JD, Noelle RJ, *et al.* CD40–CD40 ligand interactions in experimental allergic encephalomyelitis and multiple sclerosis. *Proc Natl Acad Sci U S A* 1996; 93:2499–2504.
- 124 Laman JD, van Meurs M, Schellekens MM, *et al.* Expression of accessory molecules and cytokines in acute EAE in marmoset monkeys (*Callithrix jacchus*). *J Neuroimmunol* 1998; 86:30–45.
- 125 Girvin AM, Dal Canto MC, Miller SD. CD40/CD40L interaction is essential for the induction of EAE in the absence of CD28-mediated co-stimulation. *J Autoimmun* 2002; 18:83–94.
- 126 Oppmann B, Lesley R, Blom B, *et al.* Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 2000; 13:715–725.
- 127 Natarajan C, Bright JJ. Curcumin inhibits experimental allergic encephalomyelitis by blocking IL-12 signaling through Janus kinase-STAT pathway in T lymphocytes. *J Immunol* 2002; 168:6506–6513.
- 128 Becher B, Durell BG, Noelle RJ. Experimental autoimmune encephalitis and • inflammation in the absence of interleukin-12. *J Clin Invest* 2002; 110:493–497.
IL-12 promotes EAE by the induction of autoantigen-reactive Th1 cells. The authors of this publication provide strong evidence in engineered mice that several of the activities in EAE attributed to IL-12 are actually exerted by IL-23. Both heterodimer cytokine shares the p40 subunit, which is recognized by many anti-IL-12 antibodies tested in EAE (see Ref. [126]).
- 129 Soos JM, Mujtaba MG, Schiffenbauer J, *et al.* Intramolecular epitope spreading induced by staphylococcal enterotoxin superantigen reactivation of experimental allergic encephalomyelitis. *J Neuroimmunol* 2002; 123:30–34.
- 130 de Vos AF, van Meurs M, Brok HP, *et al.* Transfer of central nervous system •• autoantigens and presentation in secondary lymphoid organs. *J Immunol* 2002; 169:5415–5423.
The progressive diversification of the repertoire of autoreactive T cells (epitope spreading) contributes to the chronicity of EAE/MS. It is unknown however, where T-cell activation to spreading epitopes takes place. Is it within the CNS (Ref. [90]) or outside the CNS in the cervical lymphoid organs that drain the CNS? This publication shows clusters of myelin antigen-loaded APCs and T cells in cervical lymph nodes of EAE-affected monkeys, suggesting that T-cell activation takes place.
- 131 Weir CR, Nicolson K, Backstrom BT. Experimental autoimmune encephalomyelitis induction in naive mice by dendritic cells presenting a self-peptide. *Immunol Cell Biol* 2002; 80:14–20.
- 132 Elhofy A, Kennedy KJ, Fife BT, *et al.* Regulation of experimental autoimmune encephalomyelitis by chemokines and chemokine receptors. *Immunol Res* 2002; 25:167–175.

Modelling of multiple sclerosis: lessons learned in a non-human primate

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The many, highly specific, biological therapies for immune-based diseases create a need for valid preclinical animal models. The wide immunological gap between human beings and laboratory mouse or rat models makes many disease models in these species invalid. In this review, we report a non-human-primate model of chronic multiple sclerosis (MS)—experimental autoimmune encephalitis (EAE) in the common marmoset (*Callithrix jacchus*)—that can help bridge this wide gap. The genetic and immunological similarity of marmosets and human beings and the clinical and neuropathological similarity of the EAE model to MS provide a unique experimental platform for research into basic immunopathogenetic mechanisms and for the development of more effective treatments for MS.

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Multiple sclerosis (MS) is a chronic progressive disease of the brain and spinal cord. The main pathological characteristic of the disease and the presumed cause of the neurological deficit is the lesion, a focally demyelinated area with a variable degree of inflammation, axonal pathology, and glial-scar formation.¹ In about 80–90% of patients with MS, the disease starts with alternating episodes of neurological impairment (relapse) and full or partial recovery (remission). In most patients, after a variable length of time, this course is followed by a secondary progressive phase where recovery no longer occurs. A few patients with primary progressive MS have irreversible worsening of clinical signs from disease onset. In a small but significant subset of patients, the disease starts as an acute inflammatory demyelinating disease fulfilling the criteria for acute disseminated encephalomyelitis, which may later develop into MS.² The clinical range of MS also includes rare variants such as Balo's concentric sclerosis and neuromyelitis optica (Devic's disease) in which the pathology is largely restricted to the optic nerve and the spinal cord.

Mainly because of the highly improved imaging techniques in the past two decades, research in patients has been intensified. Moreover, research in sophisticated animal models has greatly improved our understanding of the pathogenetic mechanisms in MS.³ Excellent overviews of the current knowledge have been published recently.^{4,5} Although the trigger of MS is still unknown, genetic and environmental factors seem to interact in the induction of the disease. Most MS investigators agree that once initiated, the immune system has a significant effect on the disease course.⁶ However, the precise determinants of the chronic

inflammatory course and the factors that underlie the neurological deficit are still poorly understood.

MS as an autoimmune disorder

MS may be caused by a combined cellular and humoral autoimmune attack on myelin sheaths and possibly axons. Several facts have contributed to the concept that MS is an autoimmune disease, such as the association with various regulatory genes of the immune response,⁷ the presence of oligoclonal immunoglobulin species in CSF points to intrathecal expansion of specific B-cell clones,⁸ and the immunopathology of the lesions.^{9,10} Further support comes from the immunopathological similarity of MS with the autoimmune animal model EAE (experimental autoimmune encephalomyelitis) in rodents¹¹ and primates.¹² However, the substantial dissimilarities between MS and EAE models have raised doubts about the autoimmune origin of MS.¹³ Notably, many of the EAE models in rodents present as a rapidly progressing monophasic disease with clinical and pathological findings that are more reminiscent of acute disseminated encephalomyelitis than chronic relapsing MS. However, exceptions do exist, such as the elegant EAE model in Biozzi/ABH mice immunised with spinal-cord homogenate.¹⁴

In this article, we review a new non-human-primate model for chronic MS in common marmosets that approximates the human disease in many ways. The clinical and neuropathological similarity with chronic MS makes this a highly useful model to test scientific hypotheses on the critical pathogenetic mechanisms and for the development of more effective therapies. This model can very usefully bridge the considerable gap between rodent EAE models and MS.

A clinical MS look-alike?

The common marmoset (*Callithrix jacchus*) is a small monkey—adults weigh 300–400 g—from the Amazon delta, South America. The neuroanatomical similarity with human

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Similarities of the marmoset EAE model and MS

General features

Neuroanatomy¹⁵
 Ratio of white matter to grey matter¹⁵
 Lymphocyte phenotype¹⁹
 Antigen capture and presentation molecules (MHC) of antigen presenting cells²⁰
 Antigen receptor of T cells²¹
 Antigen receptor of B cells and antibodies²²
 Effector molecules of the immune response²³

Disease-related features

Chronic progressive clinical course^{24,25}
 Pathomorphology of the lesion^{12,26,27}
 Immunology of the lesion²³
 MRI of the lesion^{27,28,29}
 Myelin-reactive T-cells in naive repertoire^{30,31}
 EAE-related T-cell responses to myelin antigens^{32,33,34}
 Antibody responses to myelin antigens^{32,34}

beings¹⁵ and the remarkable performance in cognitive and neurophysiology tests¹⁶ explains the popularity of this species in neurological research. These general features, added to the outbred nature and the well-established genetic¹⁷ and immunological similarity with human beings^{18–24} make the marmoset an attractive animal model for MS (panel). Clinical and neuropathological signs of MS are induced by inoculation of marmosets with CNS myelin (preferably) from patients with MS or with myelin proteins, such as myelin basic protein, proteolipid protein, or myelin-oligodendrocyte glycoprotein (MOG). To become pathogenetic these proteins need to be emulsified in a suitable formulation, such as bacterial products in mineral oil, for the induction of inflammatory disease. Despite the outbred character of our marmoset colony, 100% of myelin-immunised monkeys develop clinical EAE, albeit with substantial variation in the time of onset and severity of the neurological deficit.²⁴ This creates an attractive experimental setting for the characterisation of the immune mechanisms that regulate the induction and progression of the disease.

Neuropathology of MS

In a large multicentre analysis of active MS lesions in biopsy samples and autopsy material, four main lesion types could be discerned.¹⁰ The two prevailing lesion patterns in patients with relapsing-remitting or secondary-progressive MS displayed histological evidence of demyelination dependent

on T cells (type I) or T cells plus antibody (type II). CNS lesions in marmosets immunised with MS myelin represent all different stages of inflammatory demyelination as defined for the characteristic type II lesions in chronic MS.^{10,27} A similar analysis in the EAE marmoset model induced by recombinant human MOG revealed that the lesions were mainly of the early active type, whereas the prevalence of the initially much rarer chronic inactive lesions seemed to increase with progression of the disease.²⁸

Lesion types III and IV seem to be associated with oligodendrocyte degeneration rather than an autoimmune attack.¹¹ Ever since the initial publication, such lesions seemed to be absent in most chronic cases. However, a recent publication reported oligodendrocyte apoptosis without significant inflammation in a case of relapsing-remitting MS who died shortly after a relapse onset.³⁵ This finding has received much attention as it suggests that in common MS phenotypes, oligodendrocyte degeneration might precede white-matter inflammation and demyelination.³⁵ Such observations raise the question whether autoimmune reactions to CNS myelin may not be the cause of MS but rather a bystander response of the immune system to the release of myelin antigens induced by an elusive pathogenetic event that causes oligodendrocytes to degenerate. No such observations have been reported in the EAE models, indicating that the EAE model does not reflect the complete neuropathological range of MS.

MRI and neuropathology of marmoset EAE

An attractive feature of the marmoset EAE model is that the development of white-matter lesions in the brain can be visualised with similar MRI techniques as used in a clinical setting (figure 1). MRI of the spinal cord can be done in sedated animals, but are commonly of low quality because of breathing artefacts. Although there are no technical limitations for MRI of the mouse or rat brain, they are less useful in EAE because of the low amount of brain white matter. With the help of quantitative MRI techniques applied at regular intervals after EAE induction with recombinant human MOG, early changes in white matter that give rise to lesions can be detected and quantified.²⁸ The immunological similarity of marmosets and human beings implies that MRI-detectable brain white-matter lesions can be characterised with the same markers as used for the staging of lesions in MS.^{27,36} Also the MRI correlates of histologically defined lesion (figure 2) stages can be examined with these techniques.

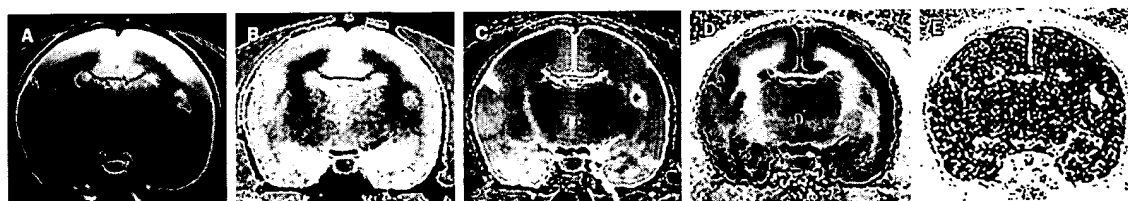


Figure 1. MRI of recombinant-human-MOG-induced EAE in marmosets. High contrast T2-weighted images (A) are used to visualise abnormalities in white matter. The specificity of MRI can be increased by using T1 (B) and T2 (C) relaxation-time images and magnetisation transfer (D) images. Images created by subtraction of images recorded before and after intravenous injection of gadolinium-DTPA 300 $\mu\text{m/kg}$ (E) visualise the permeability of the blood-brain barrier.

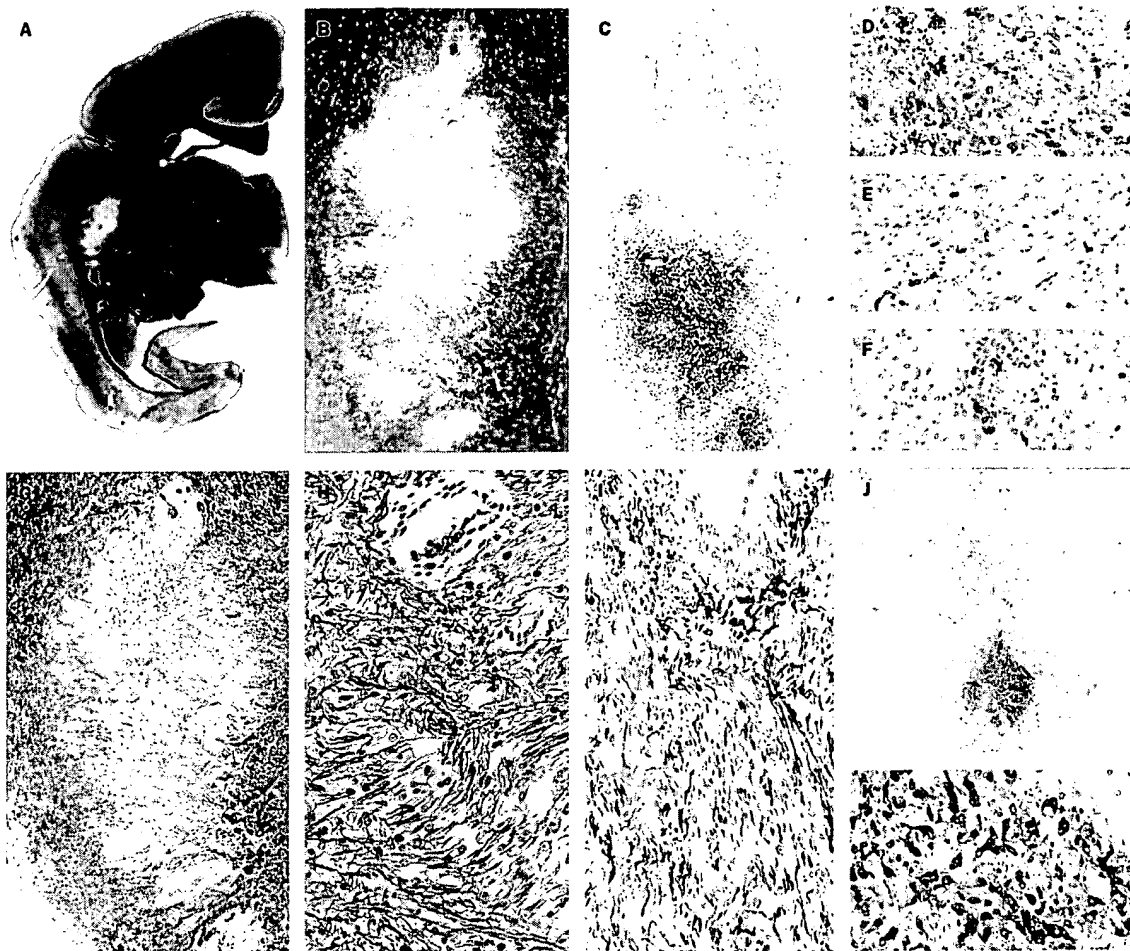


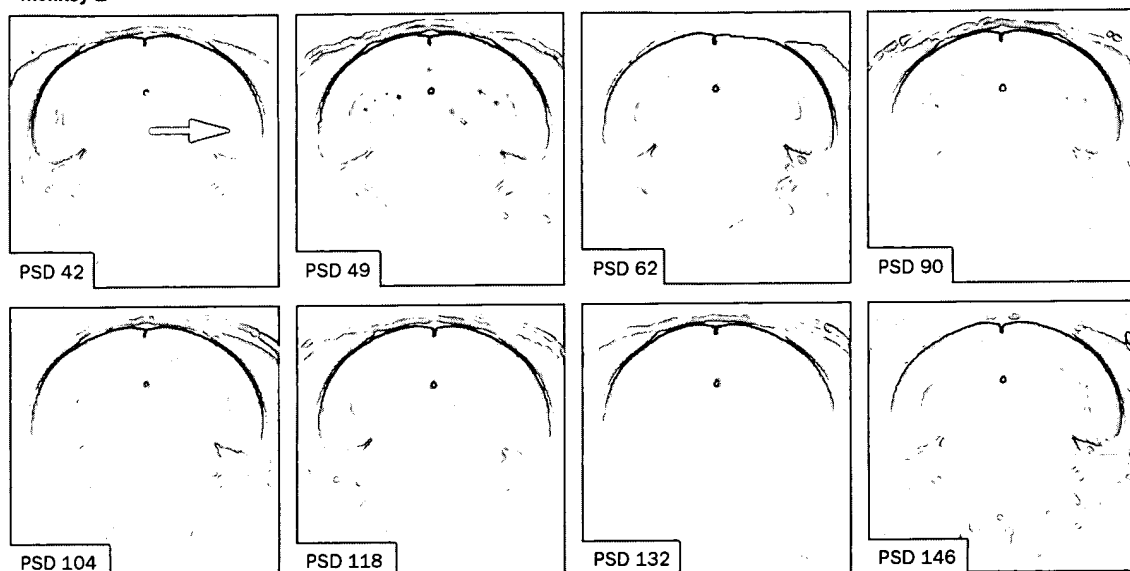
Figure 2. Histological characterisation of the MRI abnormality in figure 1. Klüver-Barerra stain for myelin shows a demyelinated lesion within the brain white matter (A). Staining for proteolipid protein (PLP) reveals complete loss of PLP in the lesion (B; x 25). In the lower part of the lesion large numbers of MRP14-positive macrophages are present (C; x 25). A higher magnification of this part of the lesion shows the presence of PLP-positive degradation products within macrophages likely derived from phagocytosed myelin (D; x 580). CD3-positive T-lymphocytes (E) and CD20-positive B-lymphocytes (F) are scattered throughout the lesions (x 125). Loss of axonal density due to oedema and cellular infiltration is seen by Bielschowsky silver stain (G; x 25). A higher magnification reveals that acute axonal degeneration in this area is relatively minor (H; x 195). Expression of non-phosphorylated neurofilaments is increased in the lesions indicating the presence of demyelinated axons (I; x 150). Staining for inducible nitric oxide synthase (iNOS) as a marker for NO radical production is seen in the centre of the lesion (J; x 125). A higher magnification reveals that iNOS is present in macrophages/microglia (K; x 160).

Axonal pathology

The association between the total volume or number of T2 lesions within the CNS white matter and the neurological deficit in MS patients is rather weak. A similar clinico-radiological paradox is sometimes observed in the marmoset EAE model, as monkeys that have the same neurological score can have completely different loads of MRI detectable brain lesions, or monkeys with more lesions can have less severe clinical signs (figure 3).³² This paradox has raised the question whether the most prominent pathological features of lesions, being inflammation and demyelination, are the direct cause of the neurological deficit. In a seminal publication, Trapp and colleagues³⁷ showed that substantial destruction of axons could already be observed in early lesion stages.

Similar to chronic MS, axonal pathology is prominent in early lesion stages in the marmoset EAE model. We observed in early demyelinated lesions that presented with active inflammation on MRI, clear staining with antibodies against amyloid precursor protein (APP; figure 4) and non-phosphorylated neurofilaments.²⁶ This pattern is reminiscent of the pathology in MS lesions.³⁸ Transected axons were not immediately evident from traditional silver staining of these lesions. Because these features were typically absent from late active demyelinated lesions,²⁶ early axonal damage may be reversible and a direct reaction to acute inflammation. Silver staining of chronic inactive lesions revealed substantial destruction of axons.^{37,38} Axonal pathology can be influenced in marmosets by the use of other non-specific proinflammatory agents, such as heat-killed *Bordetella pertussis* or the pertussis toxin during immunisation.^{25,27}

Monkey 1



Monkey 2

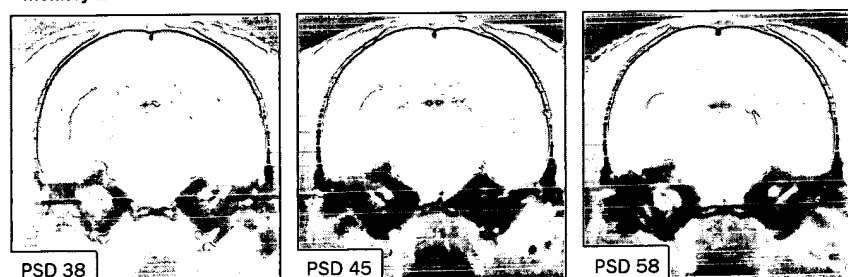


Figure 3. The discrepancy between data obtained by clinical observation and brain MRI. Monkey 1: a brain lesion of about 2 mm² (arrow) is present as is a clear neurological deficit (ataxia) at the time of MRI at postsensitisation day (PSD) 42. Monkey 2: there were no clear neurological signs at PSD 38 but there were at least eight large lesions 2–30 mm². Progression to paraplegia occurred after PSD 100 in monkey 1, but just 58 days in monkey 2.

Genetics and environmental factors in MS and EAE

The strongest genetic influence on MS susceptibility in the human population is exerted by the major histocompatibility complex (MHC) class II region, specifically mapping to the *HLA-DRB1*1501* allele. The protein products of MHC class II genes serve as antigen-capture and presentation molecules of antigen presenting cells. Similarly, susceptibility to EAE in marmosets maps to the *Caja-DR* locus, which encodes the marmoset equivalents of *HLA-DR* molecules. The *Caja-DRB1*W1201* allele, which seemed to be functionally expressed in all tested individuals from at least four outbred colonies,^{17,20} emerged as a dominant susceptibility factor to EAE.³² This genetic association could be immunologically explained, because *Caja-DRB1*W1201* functions as a major restriction element for the activation of CD4 T cells specific for the encephalitogenic MOG_{14–36} peptide.³² This idea was confirmed by the observation that CD4 T-cell lines of this specificity induce early pathological signs of EAE (perivascular cuffs) when transferred into naive MHC compatible recipients.³⁰

The idea that MS is caused by a viral infection of genetically susceptible individuals is mainly based on epidemiological and demographic data, but may be supported by the therapeutic benefit of interferon beta in patients with MS. Also the predominance of CD8 T cells in lesions,^{39,40} among which a high frequency of cells reactive with common herpes viruses, such as cytomegalovirus and Epstein-Barr virus,⁴¹ suggests that intrathecal antiviral immune reactions may play a part in MS pathogenesis. Bacterial infections are not commonly regarded as potential triggers of MS, although these may have an effect on its course.

Several specific viruses continue to be linked to MS, either because they express immunologically similar structures (mimicry motifs) as myelin antigens (cytomegalovirus and Epstein-Barr virus), or because they can substantially modulate co-stimulatory signals that lower the threshold for autoimmunity (Epstein-Barr virus). Others, such as human herpes virus 6, may have earned their place in the gallery of candidate triggers of MS by their over-

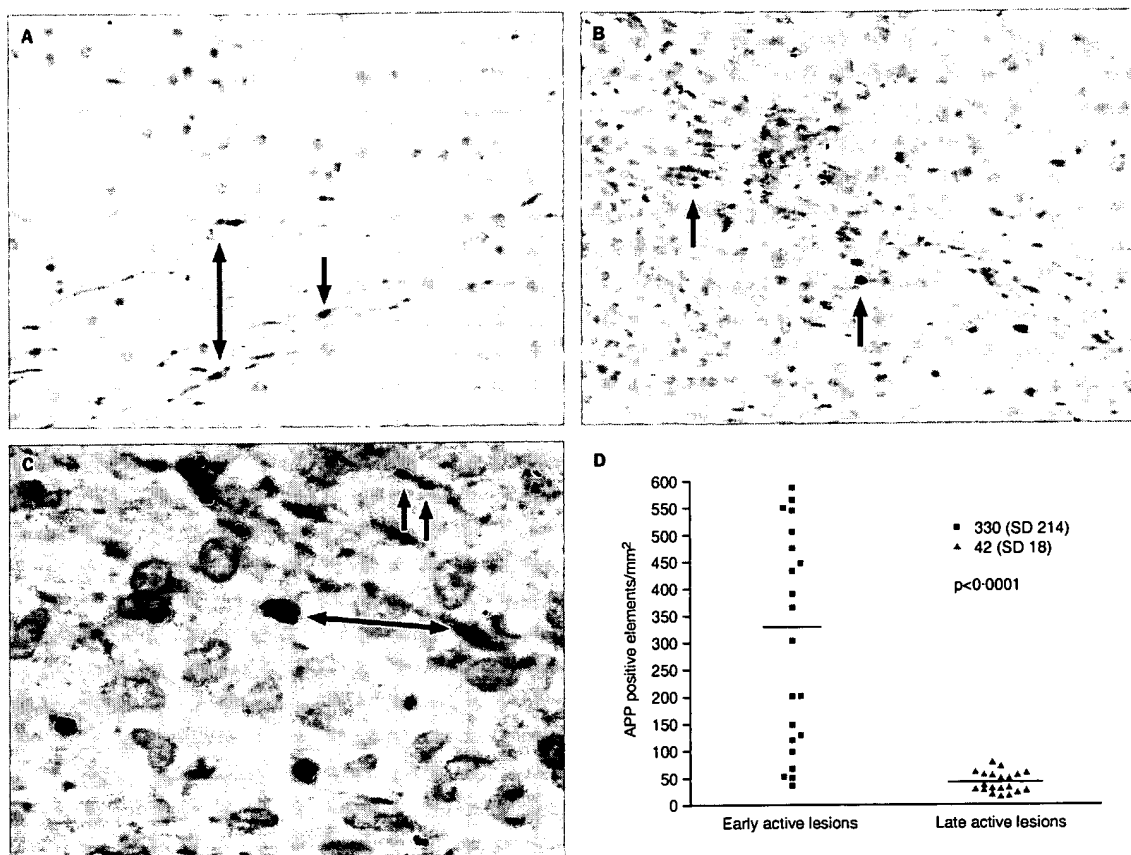


Figure 4. Early axonal pathology in EAE-affected marmosets. Arrows point to the positive staining of axons in MS (A [$\times 100$]) and rhMOG-induced marmoset EAE (B [$\times 40$]; C [$\times 100$]) with antibody to human APP. The same staining of CNS from human myelin-immunised marmosets shows that APP staining is present in early active but not in late active demyelinating lesions. Figure courtesy of Antonio Uccelli MD (Department of Neurosciences Ophthalmology and Genetics, University of Genoa, Italy).

representation in brains of patients with MS. Clinical and epidemiological data highlight Epstein-Barr virus as an important cofactor in the induction or course of MS.⁴² Marmosets can be infected with gamma herpes viruses related to Epstein-Barr virus.⁴³ It is tempting to test whether, by experimental infection with such viruses, the induction of encephalitogenic autoimmune reactions can be accurately modelled in a species that is genetically and immunologically closely related to human beings.

Pathogenetic mechanisms in the initiation of EAE in the common marmoset

The immunological mechanisms that operate in the initiation of MS lesions are rarely seen and therefore less intensively investigated. For this reason we have investigated the variety of lesions in the marmoset EAE model in more detail. A combined histological and immunohistochemical analysis has revealed high immunological activity in lesions. Similar to many rodent EAE models, we detected various activated immune cells localised within lesions, including macrophages, T cells, and B cells.⁴³ Moreover, many immune-effector molecules have been detected, such as

autoantibodies,⁴² complement factors and inhibitors (van Beek and colleagues, unpublished), cytokines and matrix metalloproteases,²³ and a novel myelencephalon-specific protease.⁴⁴

Common abnormalities observed in the (affected and normal appearing) CNS white matter of EAE-affected marmosets are the loss of tight adhesion between the normally compact myelin lamellae and the formation of myelin vesicles.⁴⁵ The mechanisms that cause the increase of polyunsaturated membrane phospholipids and negatively charged phosphatidylserine are unclear, but these changes result in decreased intermembrane adhesion. In EAE, autoantibodies may spread more easily through the loose myelin structure and thus may have better access to myelin antigens.⁴⁶

Antimyelin antibodies

The association of early pathological changes in myelin with the localisation of autoantibodies specific for MOG suggests a causal relation.¹² Indeed, the induction of demyelination and clinical signs in this model depends on the presence of antibodies against the glycoprotein.^{47,48}

These are important findings in view of the growing awareness that the presence of MOG autoantibodies may be an important predictive⁴⁹ and pathogenetic⁵⁰ factor in chronic MS. Predictably, complexes formed between antibodies and myelin surface exposed MOG (opsonisation) will activate complement factors, leading to the release of chemotactic factors, such as C5a, that can attract lymphocytes and macrophages.⁵¹ Complement factors together with regulators of complement activity, such as decay-accelerating factor, were found in CNS lesions in EAE-affected marmosets (van Beek and co-workers, unpublished). When infiltrated macrophages recognise opsonised myelin, destruction mechanisms are induced.^{52,53} Myelin debris phagocytosed by macrophages is possibly transferred to the cervical lymph nodes and may induce new autoimmune reactions, as is suggested by the localisation of myelin-loaded phagocytic cells within the areas where activation of T cells and B cells takes place.⁵⁴

As in human beings and laboratory strains of mice and rats, T cells specific for myelin components (including myelin basic protein and MOG) and able to transfer neurological disease are part of the normal immune repertoire of marmosets.^{30,31} One of the great enigmas in MS research is how, in healthy individuals, these cells are kept dormant and why they are chronically activated in patients with MS. An attractive approach to this problem is based on Terence Wilkin's primary lesion hypothesis, namely that MS patients can be viewed as "genetically predisposed high responders to CNS myelin that is released after an antecedent pathogenic event". This hypothesis implies that any pathogenetic event that causes myelin damage is a potential trigger of MS. Whether MS develops or not depends on the capacity of the affected individual to control the reaction of the immune system to the damage. As the strongest genetic influence on susceptibility to MS maps to the MHC class II region we have focused our examination on immune reactions to myelin proteins and their regulation by MHC class II molecules.

Determinants of the disease course

The determinants of the highly variable clinical course of MS are largely unknown. Exogenous factors, such as stressful life events⁵⁵ and viral⁵⁶ or bacterial infections, with *Chlamydia pneumoniae* for example,⁵⁷ have been associated with the exacerbation of clinical signs. That the frequency of disease exacerbations declines during pregnancy and normalises after birth indicates a significant hormonal influence, probably of oestriol, in MS.^{58,59} Moreover, the emergence of new T-cell and B-cell responses to myelin antigens, a phenomenon known as epitope spreading, have been implicated as a driving force of the chronic disease course.⁶⁰⁻⁶²

The type of clinical signs of EAE in marmosets depends largely on the antigens used for disease induction. Sensitisation of marmosets to human myelin induced a relapsing-remitting, secondary-progressive disease course.^{27,63} Lesions in this model represent essentially all stages present in chronic MS.²⁷ Marmosets inoculated with the myelin basic protein develop only mild inflammatory

disease unless *Bordetella pertussis* particles are infused as well.^{32,47} This worsening of the disease by administration of bacterial products is reminiscent of the clinical worsening of MS after bacterial or viral infection. CNS demyelination, being the pathological hallmark of MS, critically depends on the presence of antibodies, such as to MOG, a quantitatively minor CNS component.⁴⁷ This hallmark finding is confirmed by the observation that marmosets sensitised to a chimerical protein of myelin basic protein and proteolipid protein develop clinical EAE only once the autoimmune reaction has spread to MOG.⁴⁸ The presence of MOG antibodies in serum seems to be a predictive factor for clinically definite MS in patients with a clinically isolated syndrome.⁴⁹

Marmosets immunised with recombinant human MOG₁₋₁₂₅ do not typically have relapsing-remitting disease, but only chronic-progressive disease.^{24,25} The lesions in this model are mostly of the early active type with persistent inflammation and demyelination. During the asymptomatic phase of this primary-progressive-like disease, which can last 2-20 weeks, brain lesions are clearly detectable with MRI, but these lesions are not expressed clinically.

Patients with MS have variable T-cell and antibody reactivity with myelin antigens, but the exact relation with the disease course is poorly understood. The influence of genetic factors is also unknown. As in some rodent models, progression of the disease in marmosets immunised with recombinant human MOG is associated with a diversification of the autoimmune reaction, a phenomenon known as epitope spreading. Unpublished data in this EAE model show a statistical relation between the broadness of the epitope reactivity of MOG-reactive T cells and the shortness of the time interval after immunisation to the onset of clinical signs (unpublished).

Advantage can be taken of the outbred character and similarity to human beings of the marmoset EAE model to examine whether this relation is based on the specific interaction of certain antigens and antigen-receptors, as we have postulated for MS.⁶⁴ Clinical EAE can be induced in 100% of randomly selected marmosets by several rounds of sensitisation to MOG₁₄₋₃₆. The ubiquitous *Caja-DRB*W1201* allele is a dominant susceptibility element in this peptide-induced model.³² However, the lesions in the peptide model are mainly inflammatory and lack detectable demyelination. Recent data show that other peptides, such as MOG₃₄₋₅₆ and MOG₇₄₋₉₆, encompass immunodominant T-cell and B-cell epitopes in immunised marmosets and rhesus monkeys. These were found to be also encephalitogenic in marmosets, although the disease course is highly heterogeneous, possibly reflecting the genetic heterogeneity of the model. T-cell responses against these peptides are likely restricted by *Caja-DRB1*03* molecules.

In conclusion, EAE in the common marmoset provides an exciting new model for translational research into immunopathogenetic mechanisms in MS that bridges the wide immunological gap between rodent models and the human disease.⁶⁵ The marmoset EAE model can be of strategic importance for the preclinical selection of reagents that are being developed for intervention in

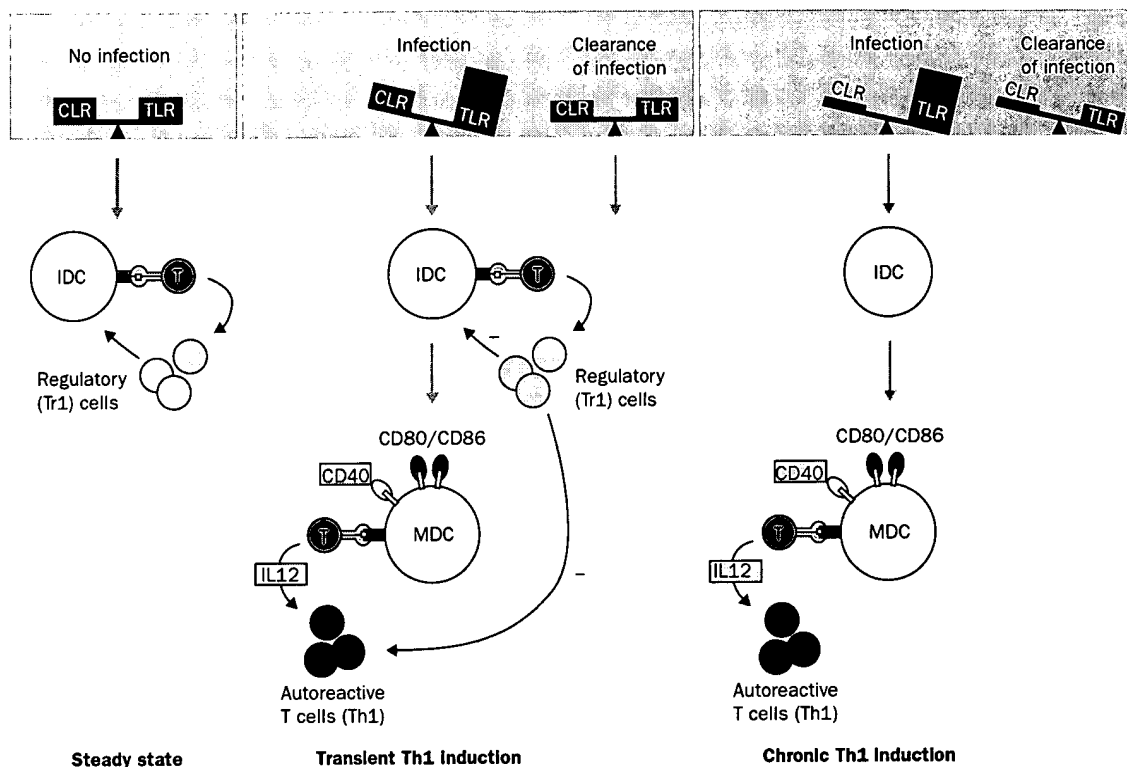


Figure 5. Regulation of dendritic cell maturation by C-type lectin (CLR) and toll-like receptors (TLR). In a healthy individual, immature dendritic cells (IDCs) secure non-responsiveness of the autoreactive T-cells in the normal repertoire by induction of regulatory T cells. This steady-state situation is maintained because DC maturation inducing signals via TLR are balanced by maturation inhibiting signals via CLR. Pathogens disturb the steady state by giving maturation inducing signals via TLR. Mature DC (MDC) induce autoreactive Th1 cells by presentation of self antigens and co-stimulation by secreted (interleukin 12) or surface-expressed (CD40) molecules. Restoration of the steady-state situation after clearance of the infection depends on the capacity of CLR to balance TLR. Conceptually, chronic induction of autoreactive Th1 cells can occur when the CLR countersignal is disturbed.

immunopathogenetic processes.^{66,67} In this way the model can help to accelerate the development of a more effective treatment for MS.

Antigen presenting cells in chronic EAE

Myelin-reactive CD4 Th1 cells are generally thought to initiate the broad cascade of pathophysiological reactions that give rise to inflammatory demyelination within the CNS of patients with MS.⁶⁸ However, therapies aimed at the physical or functional elimination of this cell-type in advanced MS have had disappointing results, despite their efficacy in EAE models.⁶⁹ Intravenous recombinant human MOG—given to prevent EAE as observed with other myelin antigens in rodent EAE models⁷⁰—induces a fatal Th2-mediated autoimmune reaction in marmosets.⁷¹ Therapies that do not target autoreactive CD4 Th1 cells themselves, but rather prevent their activation may circumvent this problem.

Tolerance induction as a permanent therapy for MS

Autoreactive Th1 cells are induced within lymphoid organs by mature dendritic cells, which present self-peptides on their MHC class II molecules. The full maturation of the autoreactive T cells depends on the simultaneous production

of essential co-stimulatory factors by the dendritic cell, which can be surface exposed, such as CD40 and CD80/CD86, or secreted, such as interleukin 12.⁷² That antibodies for CD40 or the interleukin 12p40 subunit protect marmosets immunised with myelin and recombinant human MOG, illustrates the relevance of these factors in the pathogenetic process^{73–75} (unpublished).

The ultimate aim of MS research is to re-inforce immune tolerance to CNS antigens. Such therapies should be based on a firm understanding of the mechanisms that control tolerance and immunity. Because of its similarity to human MS, the marmoset EAE model is very suitable for this line of research.

Critical to the functioning of the immune system is its capacity to distinguish “infectious non-self signals” by pathogens that should lead to immunity from “non-infectious self signals” by self-antigens that induce tolerance.⁷⁶ There is accumulating evidence that antigen-presenting cells present in lymphoid organs as well as the target organ play an important part in this process.⁷⁷ T cells—getting self antigen from immature or semimature dendritic cells, which lack expression of co-stimulatory molecules such as CD40 and CD80/CD86, and fail to produce interleukin 12—acquire the

regulatory capacity needed to maintain tolerance.^{72,78,79} This has led us to hypothesise that the regulation of dendritic-cell maturation is an important component of the control mechanisms regulating autoimmunity and tolerance. The underlying cell biological mechanisms have been discussed in detail elsewhere.^{80,81}

Dendritic-cell maturation is regulated by a dynamic crosstalk of two antigen receptor families, namely toll-like receptors and C-type lectin receptors.⁸⁰ Toll-like receptors are a highly conserved family of receptors for conserved molecular structures on pathogens, known as pathogen-associated molecular patterns. Dendritic cells receive stimulatory signals needed for their maturation via toll-like-receptor binding of pathogen associated molecular patterns, with or without engagement of cell surface molecules, such as CD40.⁸² Examples of ligands with a proven role in EAE are mycobacteria, being an essential component of the antigen formulation for EAE induction that stimulate via TLR2 and TLR4,⁸³ peptidoglycan, stimulating via TLR2 (unpublished), and CpG oligonucleotides, stimulating via TLR9.⁸⁴ The natural ligands of C-type lectin receptors are carbohydrate epitopes present on self-proteins and pathogens. C-type lectin receptors endocytose bound glycoproteins and process these for presentation to T cells.⁸⁰ Because certain C-type lectin receptors, such as DC-SIGN, do not deliver signals for maturation to the dendritic cell, antigens captured by those receptors evade a neutralising immune reaction⁸⁵ and may induce tolerance.⁸⁶

Self-glycoproteins sampled via C-type lectin receptors can thus counterbalance dendritic-cell maturation signals via toll-like receptors leading to the induction of regulatory T cells (Tr1; figure 5). Tr1 cells can maintain specific tolerance by keeping the autoreactive Th1 cells⁷⁸ and dendritic cells⁸⁷ quiescent. Pathogens delivering "danger signals"⁸⁸ to the immune system via toll-like receptors can disrupt this homeostatic control mechanism by the induction of dendritic-cell maturation. This situation occurs in the EAE model when myelin antigen is inoculated with mycobacteria in oil. The matured dendritic cells can present the co-injected self-antigen to autoreactive Th1 cells, which incite a cascade of pathophysiological reactions towards the CNS. When the toll-like receptor stimulus is cleared the balance of C-type lectin receptors and toll-like receptors is restored and the disease goes into remission, possibly because new Tr1 cells are being induced. The persistent activation of autoreactive T cells in chronic inflammatory diseases may be caused by failure of this type of control mechanism.⁸¹ For example, changes in the normal glycosylation of self glycoproteins can affect their binding to C-type lectin receptors thus creating a reduced capacity to regain control over the induction of autoreactive T cells after an infection has been cleared. A classical example illustrating this principle are the rheumatoid factors in patients with rheumatoid arthritis, which are autoantibodies directed against the abnormally glycosylated (α galactosyl) Fc tail of serum IgG molecules.^{89,90} Some articles report on the low activity of glycosyltransferases,⁹¹ enzymes catalysing protein glycosylation and abnormal glycosylation of CSF glycoproteins⁹² in MS. Because of the outbred character and

their immunological proximity to human beings, marmosets are particularly useful to investigate this mechanism in detail.

Marmoset EAE model and therapy development

There is a long list of experimental therapies for MS that showed promising effects in rodent EAE models but proved ineffective or even detrimental in patients.⁹³ Different factors contribute to this frustrating situation.

New therapeutic concepts are commonly derived from experimental observations in animal models. However, the extrapolation of experimental data obtained in aseptically raised mice from genetically homogeneous laboratory strains to the heterogeneous human population has been hampered by the wide microbiological, genetic, and immunological gap between both species.⁶⁵ For example, the ease with which the immune system of common laboratory mice can be rendered unresponsive (tolerant) to self antigens and alloantigens because of their history of microbial infections differs to that of human beings.⁹³ The similar situation in transplantation research therefore led to the strong recommendation that the efficacy of new therapeutic ideas should first be tested in non-human primates before they can enter clinical trials.^{66,67}

To be able to use the marmoset EAE model as a preclinical test system for new therapies, diagnostic tools for longitudinal monitoring of the pathogenetic process had to be developed. As in the clinical setting, the effect of a new therapy is determined on the basis of autoimmune parameters recorded in peripheral blood in combination with MRI of the brain white matter. The great advantage of the marmoset over rodents for MRI studies is the much higher ratio of white to grey matter, which is much closer to that of human beings.¹⁸ However, the generation of meaningful MRI from spinal cord is still hampered by technical problems related to the small size of marmosets. The number and spatial distribution of lesions with or without blood-brain barrier leakage can be determined by routine use of MRI based on T2-weighted and contrast-enhanced T1-weighted images. The low specificity of these routine images has been substantially improved by the implementation of quantitative MRI, which was generated by plotting T2 and T1 relaxation times or magnetisation transfer ratios.²⁸

A useful technique for the day-to-day monitoring of disease progression uses urine analysis with nuclear-magnetic-resonance spectroscopy. Data obtained in EAE-affected common marmosets and patients with MS reveal disease-related changes in the chemical composition of urine.⁹⁴ The difference is mainly determined by peaks in the aliphatic part of the proton spectrum, between 0.5–3.5 parts per million). The future identification of the excreted chemical entities should reveal whether these are related to the urinary myelin-basic-protein like material that has been found in urine from patients with MS by Whitaker and co-workers.⁹⁵

The application of these techniques in marmosets should ideally have been validated with interferon beta, a registered drug for MS with a modest clinical effect in relapsing-remitting and secondary-progressive disease, but with

Search strategy and selection criteria

References for this review were selected from articles (published after 1985) in the PubMed database and from the private files of the first author. Search terms included "multiple sclerosis", "encephalomyelitis", "marmoset", "non-human primate", "genetics" and "immunology". The final reference list was generated on the basis of primary publications and reviews relevant to the topics covered in the review.

substantial effects on MRI.^{96,97} However, the cross-reactivity of human interferon β with marmoset peripheral blood mononuclear cells as assessed by the induction of interferon γ is less than 10% (unpublished). Hence, we have used new therapeutic antibodies against critical factors for the induction of the encephalitogenic Th1 cells, such as CD40 and interleukins 12 and 23. First we tested, in placebo-controlled experiments, the prophylactic treatment of marmosets immunised with MOG with antibodies directed against human CD40 or the shared interleukin-12p40 subunit of interleukins 12 and 23.^{73–75} Both antibodies protected against EAE. In another set of experiments we showed that the intravenously injected interleukin-12p40 antibody suppresses the enlargement and increment of T2 signal intensity of pre-existing brain lesions in marmosets immunised with recombinant human MOG.

In conclusion, the marmoset EAE model is a useful preclinical test system for new therapies in MS, especially for those with highly species-specific biological reagents.

Conclusion

As the poor accessibility of the CNS limits the possibilities for direct research in patients with MS, valid animal

models are essential for our understanding of MS. Such knowledge is indispensable for the development of effective therapies against MS. Although many features of the MS immunopathogenesis have been elegantly modelled in inbred strains of rats and mice, successful therapeutic interventions in these models have shown limited predictive value for clinical success. However, similar to the situation in transplantation research, non-human primate models can help to bridge the considerable genetic and immunological gap between rodents and human beings.⁶⁶ The clinical and neuropathological proximity of the marmoset EAE model to human MS creates a platform for translational research into major questions in MS.

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Authors' contributions

B'tH prepared the initial outline of the review. JDL wrote the part on CNS immunology, JB wrote the part on EAE neuropathology, EB wrote the sections on MRI, and YvK wrote the part on innate immunology. RQH reviewed the similarities between marmoset EAE and MS. B'tH, JDL, and RQH have edited the review. EB, JB, YvK, and B'tH contributed original figures.

Conflict of interest

We have no conflict of interest.

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References

- Lassmann H. Pathology of multiple sclerosis. In: Livingstone C, ed. *McAlpine's multiple sclerosis*. 3rd edn. London: Churchill Livingstone, 1998: 323–57.
- Schwarz S, Mohr A, Knauth M, Wildemann B, Storch-Hagenlocher B. Acute disseminated encephalomyelitis: a follow-up study of 40 adult patients. *Neurology* 2001; 56: 1313–18.
- Owens T, Wekerle H, Antel J. Genetic models for CNS inflammation. *Nat Med* 2001; 7: 161–66.
- Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. *N Engl J Med* 2000; 343: 938–52.
- Compston A, Coles A. Multiple sclerosis. *Lancet* 2002; 359: 1221–31.
- Wekerle H. Immunology of multiple sclerosis. In: Compston A, Ebers G, Lassmann H, McDonald L, Matthews B, Wekerle H, eds. *McAlpine's multiple sclerosis*. London: Churchill Livingstone, 1998: 379–407.
- Dyment DA, Ebers GC, Sadovnick AD. Genetics of multiple sclerosis. *Lancet Neurol* 2004; 3: 104–10.
- Fishman RA. Cerebrospinal fluids in diseases of the nervous system. Philadelphia: WB Saunders, 1992.
- Brosnan CF, Raine CS. Mechanisms of immune injury in multiple sclerosis. *Brain Pathol* 1996; 6: 243–57.
- Lucchinetti C, Brück W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol* 2000; 47: 707–17.
- Lassmann H, Brück W, Lucchinetti C. Heterogeneity of multiple sclerosis pathogenesis: implications for diagnosis and therapy. *Trends Mol Med* 2001; 7: 115–21.
- Raine CS, Cannella B, Hauser SL, Genain CP. Demyelination in primate autoimmune encephalomyelitis and acute multiple sclerosis lesions: a case for antigen-specific antibody mediation. *Ann Neurol* 1999; 46: 144–60.
- Compston A. Revisiting the pathogenesis of multiple sclerosis revisited. *Int MS* 2003; 10: 29–31.
- Baker D, O'Neill JK, Gschmeissner SE, Wilcox CE, Butter C, Turk JL. Induction of chronic relapsing experimental allergic encephalomyelitis in Biozzi mice. *J Neuroimmunol* 1990; 28: 261–70.
- Stephan H, Baron G, Schwedtfeger WK. The brain of the common marmoset (*Callithrix jacchus*): a stereotactic atlas. Berlin: Springer Verlag, 1980.
- Scott L, Pearce P, Fairhall S, Muggleton N, Smith J. Training nonhuman primates to cooperate with scientific procedures in applied biomedical research. *J Appl Anim Welf Sci* 2003; 6: 199–207.
- Bontrop RE, Otting N, de Groot NG, Doxiadis GG. Major histocompatibility complex class II polymorphisms in primates. *Immunol Rev* 1999; 167: 339–50.
- 't Hart BA, Losen M, Brok HPM, de Baets MH. Chronic Diseases. In: Wolfe-Coote SP, ed. *The laboratory primate*. London: Elsevier, 2004.
- Brok HP, Hornby RJ, Griffiths GD, Scott LA, Hart BA. An extensive monoclonal antibody panel for the phenotyping of leukocyte subsets in the common marmoset and the cotton-top tamarin. *Cytometry* 2001; 45: 294–303.
- Antunes SG, de Groot NG, Brok H, et al. The common marmoset: a new world primate species with limited Mhc class II variability. *Proc Natl Acad Sci USA* 1998; 95: 11745–50.
- Uccelli A, Oksenberg JR, Jeong MC, et al. Characterization of the TCRB chain repertoire in the new world monkey *Callithrix jacchus*. *J Immunol* 1997; 158: 1201–07.
- von Büdingen HC, Hauser SL, Nabavi CB, Genain CP. Characterization of the expressed immunoglobulin IGHV repertoire in the new world marmoset *Callithrix jacchus*. *Immunogenetics* 2001; 53: 557–63.
- Laman JD, van Meurs M, Schellekens MM, et al. Expression of accessory molecules and cytokines in acute EAE in marmoset monkeys (*Callithrix jacchus*). *J Neuroimmunol* 1998; 86: 30–45.
- Brok HP, Bauer J, Jonker M, et al. Non-human primate models of multiple sclerosis. *Immunol Rev* 2001; 183: 173–85.
- Genain CP, Hauser SL. Experimental allergic encephalomyelitis in the New World monkey *Callithrix jacchus*. *Immunol Rev* 2001; 183: 159–72.
- Mancardi G, Hart B, Roccatagliata L, et al. Demyelination and axonal damage in a non-human primate model of multiple sclerosis. *J Neurol Sci* 2001; 184: 41–49.
- 't Hart BA, Bauer J, Muller HJ, et al. Histopathological characterization of magnetic resonance imaging- detectable brain white matter lesions in a primate model of multiple sclerosis: a correlative study in the experimental autoimmune encephalomyelitis model in common marmosets (*Callithrix jacchus*). *Am J Pathol* 1998; 153: 649–63.
- 't Hart BA, Vogels JT, Bauer J, Brok HPM, Blezer E. Non-invasive measurement of brain damage in a primate model of multiple sclerosis. *Trends Mol Med* 2004; 10: 85–91.
- Jordan EK, McFarland HJ, Lewis BK, et al. Serial MR imaging of experimental autoimmune encephalomyelitis induced by human white matter or by chimeric myelin-basic and proteolipid protein in the common marmoset. *AJNR Am J Neuroradiol* 1999; 20: 965–76.
- Villoslada P, Abel K, Heald N, Goertsches R, Hauser SL, Genain CP. Frequency, heterogeneity and encephalitogenicity of T cells specific for myelin oligodendrocyte glycoprotein in naive outbred primates. *Eur J Immunol* 2001; 31: 2942–50.
- Genain CP, Lee-Parritz D, Nguyen MH, et al. In

- healthy primates, circulating autoreactive T cells mediate autoimmune disease. *J Clin Invest* 1994; 94: 1339–45.
- 32 Brok HP, Uccelli A, Kerlero De Rosbo N, et al. Myelin/oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis in common marmosets: the encephalitogenic T cell epitope pMOG24–36 is presented by a monomorphic MHC class II molecule. *J Immunol* 2000; 165: 1093–101.
 - 33 Uccelli A, Giunti D, Mancardi G, et al. Characterization of the response to myelin basic protein in a non human primate model for multiple sclerosis. *Eur J Immunol* 2001; 31: 474–79.
 - 34 von Büdingen HC, Tanuma N, Villoslada P, Ouallet JC, Hauser SL, Genain CP. Immune responses against the myelin/oligodendrocyte glycoprotein in experimental autoimmune demyelination. *J Clin Immunol* 2001; 21: 155–70.
 - 35 Barnett MH, Prineas JW. Relapsing and remitting multiple sclerosis: pathology of the newly forming lesion. *Ann Neurol* 2004; 55: 458–68.
 - 36 Brück W, Porada P, Poser S, et al. Monocyte/macrophage differentiation in early multiple sclerosis lesions. *Ann Neurol* 1995; 38: 788–96.
 - 37 Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. Axonal transection in the lesions of multiple sclerosis. *N Engl J Med* 1998; 338: 278–85.
 - 38 Kuhlmann T, Lingfeld G, Bitsch A, Schuchardt J, Brück W. Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. *Brain* 2002; 125: 2202–12.
 - 39 Babbe H, Roers A, Waisman A, et al. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J Exp Med* 2000; 192: 393–404.
 - 40 Bauer J, Rauschka H, Lassmann H. Inflammation in the nervous system: the human perspective. *Glia* 2001; 36: 235–43.
 - 41 Scotet E, Peyrat MA, Saulquin X, et al. Frequent enrichment for CD8 T cells reactive against common herpes viruses in chronic inflammatory lesions: towards a reassessment of the physiopathological significance of T cell clonal expansions found in autoimmune inflammatory processes. *Eur J Immunol* 1999; 29: 973–85.
 - 42 Munch M, Hvas J, Christensen T, Møller-Larsen A, Haahr S. The implications of Epstein-Barr virus in multiple sclerosis—a review. *Acta Neurol Scand Suppl* 1997; 169: 59–64.
 - 43 Rivaille P, Cho YG, Wang F. Complete genomic sequence of an Epstein-Barr virus-related herpesvirus naturally infecting a new world primate: a defining point in the evolution of oncogenic lymphocryptoviruses. *J Virol* 2002; 76: 12055–68.
 - 44 Scarisbrick IA, Blaber SI, Lucchini CF, Genain CP, Blaber M, Rodriguez M. Activity of a newly identified serine protease in CNS demyelination. *Brain* 2002; 125: 1283–96.
 - 45 Ohler B, Graf K, Bragg R, et al. Role of lipid interactions in autoimmune demyelination. *Biochim Biophys Acta* 2004; 1688: 10–17.
 - 46 Genain CP, Cannella B, Hauser SL, Raine CS. Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nat Med* 1999; 5: 170–75.
 - 47 Genain CP, Nguyen MH, Letvin NL, et al. Antibody facilitation of multiple sclerosis-like lesions in a nonhuman primate. *J Clin Invest* 1995; 96: 2966–74.
 - 48 McFarland HL, Lobito AA, Johnson MM, et al. Determinant spreading associated with demyelination in a nonhuman primate model of multiple sclerosis. *J Immunol* 1999; 162: 2384–90.
 - 49 Berger T, Rubner P, Schautzer F, et al. Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event. *N Engl J Med* 2003; 349: 139–45.
 - 50 Iglesias A, Bauer J, Litztenburger T, Schubart A, Linington C. T- and B-cell responses to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis and multiple sclerosis. *Glia* 2001; 36: 220–34.
 - 51 Frank MM, Fries LF. The role of complement in inflammation and phagocytosis. *Immunol Today* 1991; 12: 322–26.
 - 52 Dijkstra CD, De Groot CJ, Huitinga I. The role of macrophages in demyelination. *J Neuroimmunol* 1992; 40: 183–88.
 - 53 van der Laan LJ, Ruuls SR, Weber KS, Lodder IJ, Dopp EA, Dijkstra CD. Macrophage phagocytosis of myelin in vitro determined by flow cytometry: phagocytosis is mediated by CR3 and induces production of tumor necrosis factor- α and nitric oxide. *J Neuroimmunol* 1996; 70: 145–52.
 - 54 de Vos AF, van Meurs M, Brok HP, et al. Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs. *J Immunol* 2002; 169: 5415–23.
 - 55 Buljevac D, Hop WC, Reeder W, et al. Self reported stressful life events multiple sclerosis: prospective study. *BMJ* 2003; 327: 646.
 - 56 Buljevac D, Flach HZ, Hop WC, et al. Prospective study on the relationship multiple sclerosis exacerbations. *Brain* 2002; 125: 952–60.
 - 57 Buljevac D, Verkooyen RP, Jacobs BC, et al. *Chlamydia pneumoniae* and the risk for exacerbation in multiple sclerosis patients. *Ann Neurol* 2003; 54: 828–31.
 - 58 Confavreux C, Hutchinson M, Hours MM, Cortinovis-Tourniaire P, Moreau T. Rate of pregnancy-related relapse in multiple sclerosis: Pregnancy in Multiple Sclerosis Group. *N Engl J Med* 1998; 339: 285–91.
 - 59 Voskuhl RR. Gender issues and multiple sclerosis. *Curr Neurol Neurosci Rep* 2002; 2: 277–86.
 - 60 Tuohy VK, Yu M, Yin L, et al. The epitope spreading cascade during progression of experimental autoimmune encephalomyelitis and multiple sclerosis. *Immunol Rev* 1998; 164: 93–100.
 - 61 Vanderlugt CL, Begolka WS, Neville KL, et al. The functional significance of epitope spreading and its regulation by co-stimulatory molecules. *Immunol Rev* 1998; 164: 63–72.
 - 62 Goebels N, Hofstetter H, Schmidt S, Brunner C, Wekerle H, Hohlfield R. Repertoire dynamics of autoreactive T cells in multiple sclerosis patients and healthy subjects: epitope spreading versus clonal persistence. *Brain* 2000; 123: 508–18.
 - 63 Massacesi L, Genain CP, Lee-Parritz D, Letvin NL, Canfield D, Hauser SL. Active and passively induced experimental autoimmune encephalomyelitis in common marmosets: a new model for multiple sclerosis. *Ann Neurol* 1995; 37: 519–30.
 - 64 't Hart BA, Brok HP, Amor S, Bontrop RE. The major histocompatibility complex influences the ethiopathogenesis of MS-like disease in primates at multiple levels. *Hum Immunol* 2001; 62: 1371–81.
 - 65 Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *J Immunol* 2004; 172: 2731–38.
 - 66 't Hart B, Amor S, Jonker M. Evaluating the validity of animal models for research into therapies for immune-based disorders. *Drug Discov Today* 2004; 9: 517–24.
 - 67 Sachs DH. Tolerance: of mice and men. *J Clin Invest* 2003; 111: 1819–21.
 - 68 Lassmann H, Ransohoff RM. The CD4-TH1 model for multiple sclerosis: a crucial re-appraisal. *Trends Immunol* 2004; 25: 132–37.
 - 69 Wiendl H, Hohlfield R. Therapeutic approaches in multiple sclerosis: lessons from failed and interrupted treatment trials. *BioDrugs* 2002; 16: 183–200.
 - 70 Miller SD, Karpus WJ. The immunopathogenesis and regulation of T-cell-mediated demyelinating diseases. *Immunol Today* 1994; 15: 356–61.
 - 71 Genain CP, Abel K, Belmar N, et al. Late complications of immune deviation therapy in a nonhuman primate. *Science* 1996; 274: 2054–57.
 - 72 Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol* 2002; 23: 445–49.
 - 73 Boon L, Brok HP, Bauer J, et al. Prevention of experimental autoimmune encephalomyelitis in the common marmoset (*Callithrix jacchus*) using a chimeric antagonist monoclonal antibody against human CD40 is associated with altered B cell responses. *J Immunol* 2001; 167: 2942–49.
 - 74 Laman JD, 't Hart BA, Brok H, et al. Protection of marmoset monkeys against EAE by treatment with a murine antibody blocking CD40 (mu5D12). *Eur J Immunol* 2002; 32: 2218–28.
 - 75 Brok HP, Van Meurs M, Bleser E, et al. Prevention of experimental autoimmune encephalomyelitis in common marmosets using an anti-IL-12p40 monoclonal antibody. *J Immunol* 2002; 169: 6554–63.
 - 76 Janeway CA Jr. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today* 1992; 13: 11–16.
 - 77 Darabi K, Karulin AY, Boehm BO, et al. The third signal in T cell-mediated autoimmune disease? *J Immunol* 2004; 173: 92–99.
 - 78 Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. *Nat Rev Immunol* 2003; 3: 253–57.
 - 79 Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol* 2003; 21: 685–711.
 - 80 Geijtenbeek TB, Van Vliet SJ, Engering A, BA TH, Van Kooyk Y. Self- and nonself-recognition by C-type lectins on dendritic cells. *Annu Rev Immunol* 2004; 22: 33–54.
 - 81 't Hart BA, van Kooyk Y. Yin-Yang regulation of autoimmunity by dendritic cells. *Trends Immunol* 2004; 25: 353–59.
 - 82 Kaisho T, Akira S. Regulation of dendritic cell function through toll-like receptors. *Curr Mol Med* 2003; 3: 759–71.
 - 83 Tsuji S, Matsumoto M, Takeuchi O, et al. Maturation of human dendritic cells by cell wall skeleton of *Mycobacterium bovis* bacillus Calmette-Guérin: involvement of toll-like receptors. *Infect Immun* 2000; 68: 6883–90.
 - 84 Ichikawa HT, Williams LP, Segal BM. Activation of APCs through CD40 or Toll-like receptor 9 overcomes tolerance and precipitates autoimmune disease. *J Immunol* 2002; 169: 2781–87.
 - 85 Van Kooyk Y, Geijtenbeek TB. DC-SIGN: escape mechanism for pathogens. *Nat Rev Immunol* 2003; 3: 697–709.
 - 86 Mahnke K, Knop J, Enk AH. Induction of tolerogenic DCs: 'you are what you eat'. *Trends Immunol* 2003; 24: 646–51.
 - 87 Fallarino F, Grohmann U, Hwang KW, et al. Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol* 2003; 4: 1206–12.
 - 88 Matzinger P. The danger model: a renewed sense of self. *Science* 2002; 296: 301–05.
 - 89 Bond A, Alavi A, Axford JS, Youinou P, Hay FC. The relationship between exposed galactose and N-acetylglucosamine residues on IgG in rheumatoid arthritis (RA), juvenile chronic arthritis (JCA) and Sjögren's syndrome (SS). *Clin Exp Immunol* 1996; 105: 99–103.
 - 90 Bond A, Alavi A, Axford JS, et al. A detailed lectin analysis of IgG glycosylation, demonstrating disease specific changes in terminal galactose and N-acetylglucosamine. *J Autoimmun* 1997; 10: 77–85.
 - 91 Orlicchio A, Sarchielli P, Gallai V, Datti A, Saccardi C, Palmerini CA. Activity levels of a beta1,6 N-acetylglucosaminyltransferase in lymphomonocytes from multiple sclerosis patients. *J Neurol Sci* 1997; 151: 177–83.
 - 92 Saso L, Valentini G, Leone MG, et al. Changes in concanavalin A-reactive proteins in neurological disorders. *J Clin Lab Anal* 1999; 13: 158–65.
 - 93 Adams AB, Williams MA, Jones TR, et al. Heterologous immunity provides a potent barrier to transplantation tolerance. *J Clin Invest* 2003; 111: 1887–95.
 - 94 't Hart BA, Vogels JT, Spijksma G, Brok HP, Polman C, van der Greef J. 1H-NMR spectroscopy combined with pattern recognition analysis reveals characteristic chemical patterns in urines of MS patients and non-human primates with MS-like disease. *J Neurol Sci* 2003; 212: 21–30.
 - 95 Whitaker JN. Myelin basic protein-like material in the urine of multiple sclerosis patients: relationships to clinical and neuroimaging changes. *Mult Scler* 1998; 4: 243–46.
 - 96 Arnason BG. Interferon beta in multiple sclerosis. *Clin Immunol Immunopathol* 1996; 81: 1–11.
 - 97 Molyneux PD, Barker GJ, Barkhof F, et al. Clinical-MRI correlations in a European trial of interferon beta-1b in secondary progressive MS. *Neurology* 2001; 57: 2191–97.

Non-invasive measurement of brain damage in a primate model of multiple sclerosis

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Early recognition of whether a product has potential as a new therapy for treating multiple sclerosis (MS) relies upon the quality of the animal models used in the pre-clinical trials. The promising effects of new treatments in rodent models of experimental autoimmune encephalomyelitis (EAE) have rarely been reproduced in patients suffering from MS. EAE in outbred marmoset monkeys, *Callithrix jacchus*, is a valid new model, and might provide an experimental link between EAE in rodent models and human MS. Using magnetic resonance imaging techniques similar to those used in patients suffering from MS pathological abnormalities in the brain, white matter of the animal can be visualized and quantified. Moreover, NMR spectroscopy, in combination with pattern recognition, offers an advanced uroscopic technique for the identification of biomarkers of inflammatory demyelination.

Multiple sclerosis (MS) is a chronic progressive inflammatory disease of the human central nervous system (CNS). There are approximately one million MS sufferers worldwide, with a prevalence in Europe and the USA of ~1 in 1000 young adults. The disease usually begins in early adulthood and progresses, in most patients, to serious and irreversible neurological disability over a period of 10–30 years. The clinical expression of MS is highly variable. In ~90% of cases, it initially presents as intermittent periods of neurological deficit followed by partial or complete recovery (relapsing or remitting MS), which, in 50% of patients, is followed by a progressive phase, during which neurological functions are progressively lost and recovery no longer occurs (secondary progressive MS). In 10% of patients, progressive neurological deficit occurs from the onset (primary progressive MS). Pathologically, MS is characterized by multiple lesions with inflammatory demyelination and a variable degree of axonal pathology and scar formation by proliferating astrocytes [1–3]. Clinical symptoms during the early phases of the disease can be decreased with immunomodulation therapies, such

as treatment with interferon- β or glatiramer acetate, probably because the symptoms are caused mainly by inflammatory demyelination. The irreversible and currently untreatable neurological disability in patients with advanced MS is probably a result of the accumulated destruction of nerve tracks (axons) [4].

Research of patients with MS, and of experimental autoimmune encephalomyelitis (EAE) in animal models, has increased our understanding of the pathogenic mechanisms involved in the formation of lesions and the induction of neurological deficit. Based upon this knowledge, a variety of new therapeutic principles have been developed and successfully tested in EAE models [5]. However, in only a few cases were the beneficial effects observed in the experimental models reproduced in clinical trials [6]. Furthermore, detrimental effects were observed, for example, following treatment with altered peptide ligands [7] or anti-tumor necrosis factor- α antibody [8].

Current disease models in rodents might not be sufficiently predictive of therapeutic success in humans, a situation that is not unique for MS [9,10]. Therefore, there is the need for an improved and more predictable animal disease model of MS. Here we describe a new model of chronic MS in the common marmoset *Callithrix jacchus*. The genetic and immunological similarities of marmosets and humans, and the genetic heterogeneity of this model, create a unique opportunity for research on the molecular mechanisms operating in the induction and progression of the disease. The clinical, anatomical and neuropathological presentation of this model reflects more closely the situation found in chronic MS than do any of the current EAE models (a systematic comparison of EAE models in rodents and non-human primates has been published previously [11,12]).

Non-human primates as models of autoimmune disease in humans

The different reaction to immunomodulatory agents of 12-week-old specific-pathogen-free (SPF) quality inbred laboratory mice and of primates or humans is probably caused by a different history of microbial infections [13].

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Outbred populations of conventionally housed non-human primates (henceforth referred to as primates) more closely reflect the immune status of the human population than do pathogen-free inbred laboratory mice, and might therefore provide a more predictive disease model [10]. Many clinical and pathological features of autoimmune diseases in humans, such as MS or rheumatoid arthritis, have been reproduced successfully in primates [5,14]. Primate disease models have proven particularly useful for the efficacy testing of biotechnology-based therapies, which, as a result of their specificity for humans, cross-react only with higher primate species.

Common marmosets as a model of immunological and neurological diseases

The common marmoset is a small monkey that has been used as an experimental model for various neuropathological disorders, such as stroke, Alzheimer's disease, Parkinsonism and MS [15–18]. The structural and functional similarity of the human and common marmoset brain [19] and the performance of this species in cognitive and behavioral studies [20] have been instrumental in its development as an *in vivo* model for neurological research.

Of importance for immunological research is the fact that marmosets usually give birth to non-identical twins or triplets, which, because they have shared the placental bloodstream *in utero* are complete bone marrow chimeras. Because the immune cells of fraternal siblings are educated in a common thymic environment, the normal alloreaction towards transferred blood cells between non-identical twins does not occur [21]. This allospecific tolerance of siblings has been used in T-cell transfer studies, with the aim of determining the exact contributions of autoreactive T cells and antibodies to the pathogenesis of EAE [22,23].

Creation of an EAE model in the common marmoset

EAE is the most widely investigated animal model of MS [12]. Two EAE models have been established in the common marmoset, which mainly represent the type II lesion pathology that is prevalent in chronic MS.

Myelin-induced EAE

Immunization, with myelin from sufferers of MS, of randomly selected monkeys from an outbred population induces a 100% incidence of EAE. [11,22,24]. This remarkably high disease incidence has a genetic basis, in that EAE is initiated in each monkey by the activation of a cytotoxic CD4⁺CD16[−]CD56⁺T-cell subset specific for a peptide from the quantitatively minor myelin glycoprotein (pMOG_{14–36}), with which EAE can be induced [25]. The presence of the peptide presenting *Caja-DRB**W1201 molecule in each immunized marmoset explains the 100% disease incidence in a genetically heterogeneous marmoset colony [26,27].

The clinical and neuropathological presentation of EAE induced with myelin derived from the brains of patients with MS closely approximates chronic MS in humans. Most commonly observed is a relapsing or remitting, or secondary progressive disease [28]. The CNS white matter lesions in this model are characterized by infiltrates of

mononuclear cells and primary demyelination, with little axonal destruction. However, more detailed histological examination reveals axonal pathology confined to inflammatory active lesions, namely the accumulation of β -amyloid precursor protein and reduced phosphorylation of neurofilaments [29]. These signs of axonal 'suffering' are probably transient because they are absent in inflammatory inactive lesions. By contrast, the chronic inactive lesions display serious destruction of axons with substantial gliosis, as well as remyelination [28,30].

The immunological similarity of humans enables the immunological characterization of lesions in the EAE-affected marmoset using the same reagents as are used in patients with MS [31–33]. Infiltrated T cells and macrophages within inflammatory active lesions in the brain and spinal cord express the activation molecules CD154 (T cells), and MRP14, 27E10 and CD40 (macrophages) [28,31]. The expression within lesions of Th1 and Th2 cytokines, interferons, matrix metalloproteases, [31], and anti-MOG (myelin/oligodendrocyte glycoprotein) antibodies [30] supports the concept that inflammatory demyelination is driven by a combination of cellular and humoral autoimmune reactions to components of the CNS white matter.

The immunological mechanisms that determine the time of onset and disease duration in monkeys are unknown [5]. Damaged myelin is ingested by macrophages, which can also be found localized within the spleen and cervical lymph nodes that drain the interstitial and cerebrospinal fluids from the CNS [34]. The localization in close conjunction with T and B cells suggests that induction of new autoreactive T and B cells can occur.

RhMOG-induced EAE

The role of MOG as a primary target for the autoimmune attack on CNS myelin in MS is emphasized by the ability of anti-MOG antibodies to induce demyelination [23], in addition to the fact that these antibodies are found attached to disintegrating myelin around the axons and within lesions [35]. Autoimmunity of marmosets to MOG has emerged as a common denominator in the immunopathogenesis of EAE. For example, common marmosets immunized with MP4, a chimeric protein combining human myelin basic protein (MBP) and proteolipid protein (PLP), develop neurological signs only following the development of an autoantibody response to MOG [36]. Moreover, the dominant T- and B-cell autoreactivity in myelin-immunized marmosets is directed against MOG [25].

The most important features of the myelin-induced EAE model can be reproduced in marmosets immunized with a recombinant protein that represents the extracellular N-terminal part of human MOG (1–125; rhMOG). In rhMOG-immunized monkeys, clinical signs appear unpredictably after an asymptomatic period ranging between two and 15 weeks [5]. The predominant lesion is a sharply defined area of profound inflammatory demyelination with relatively well conserved axonal structures (Figure 1a), but chronic inactive lesions with clear axonal destruction are also evident (Figure 1b).

The rhMOG-induced EAE model is particularly suitable for the analysis of autoimmune factors that contribute

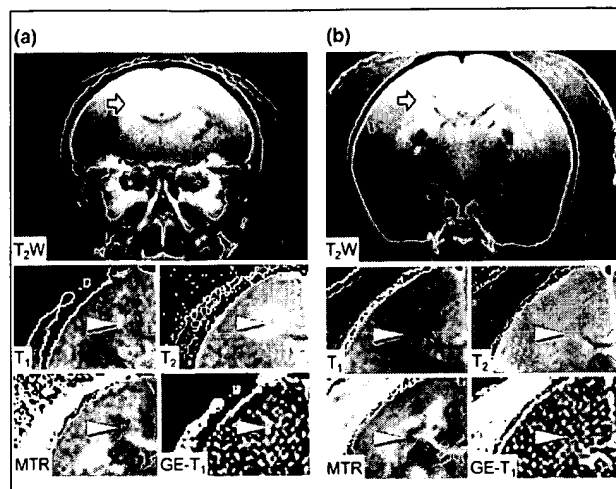


Figure 1. Magnetic resonance imaging-histology correlation of an inflammatory active and a chronic inactive lesion in a marmoset brain affected by recombinant human myelin/oligodendrocyte glycoprotein (rhMOG)-induced experimental autoimmune encephalomyelitis (EAE). (a) The inflammatory active demyelinated lesion (infiltrated by numerous MRP14-positive macrophages) is seen as a hyper-intense region on the T_2 -weighted images (T_2W) and displays increased T_2 and T_1 signal relaxation times, increased leakage of the probe gadolinium-DTPA (GE- T_1) and a reduced MTR value. (b) The inflammatory inactive lesion, in which no significant macrophage infiltrate is present, appears as a similar hyper-intense area on the T_2W image. However, T_1 and T_2 relaxation times are only marginally increased compared to the surrounding normal appearing white matter. Furthermore, this lesion shows an intermediate reduction of MTR, and no increase in GE- T_1 (and thus no blood-brain barrier leakage).

to the outbreak of clinical signs [5]. At least one immunological factor has been identified that determines the length of the asymptomatic period; the extent to which T-cell reactivity against a panel of 22-mer MOG peptides has diversified, a phenomenon known as epitope spreading. A possible explanation is that exacerbation of clinical signs occurs when specific spreading epitopes interact with the particular major histocompatibility complex (MHC) molecules that are associated with increased disease susceptibility [37,38].

Magnetic resonance imaging (MRI) of the EAE-affected marmoset brain

Inherent to this MS model are the unpredictable variations in the time of onset and the course of clinical signs among individuals. This poses considerable problems for

the statistical evaluation of efficacy tests for new therapeutic reagents in conventional two-leg placebo-controlled settings, unless large test groups are used. Instead, each animal is used as its own control, with the disease parameters monitored using non-invasive imaging techniques, both before and after treatment with the candidate therapeutic (or placebo) [9].

MRI is the preferred technique for the visualization of pathological alterations within the brain of patients with MS [39] (The different techniques are explained in Box 1). The brain white matter lesions that develop in the myelin-induced EAE model resemble those in chronic MS, and are visualized using the same MRI techniques [28,30,40]. The importance of the disease model is that MRI events in lesions can be directly correlated with histologically defined events [41,42]. For this purpose, semi-quantitative images are used, which are created by plotting the T_1 - and T_2 - relaxation times and magnetization transfer ratios (MTR) of individual lesions (Figure 1a). As an example, the MRI correlates of an early active and an inactive lesion are given in figure 1. Current studies are aimed at further differentiating lesion stages by precise calculation of the exact NMR values of individual lesions.

The development of individual brain lesions in marmosets can also be monitored precisely with serial quantitative MRI. The serially recorded MRI maps depicted in Figure 2 show that the first MRI-detectable sign of a lesion is the increased permeability of the blood-brain barrier, which appears as a focally increased T_1 -weighted signal intensity (and thus decreased T_1 relaxation time) due to the extravasation of the injected probe gadolinium-DTPA. The local accumulation of vasogenic edema causes an increased T_2 signal intensity and a moderate reduction of the MTR. The reduced density of tissue macromolecules by demyelination and axonal destruction also causes an increased T_1 signal intensity and a further decrease of the MTR.

With these clinically relevant MRI tools, the effect of a new therapeutic agent on existing brain lesions can also be determined. To validate this system, biological reagents with proven therapeutic effect are being tested in the marmoset EAE model, such as antibodies directed against human CD40 molecules and human interleukin-12p40 [43–45].

Box 1. Basic principles of MRI

Magnetic resonance imaging (MRI) is the preferred technique for the visualization of lesions in the brain and spinal cord of patients with MS. It visualizes the resonance signals of tissue protons when they are placed in a time-varying strong magnetic field. The most frequently used parameters measured in MS are the spin-lattice relaxation time (T_1) and the spin-spin relaxation time (T_2). MRI is routinely used as a tomographic imaging technique, where anatomical pictures are created of 1-mm thick tissue sections. The contrast differences between brain structures in most MRI techniques are determined by the different densities and diffusion of protons, as well as differences in relaxation times. T_2 images are sensitive to water and, because all pathological alterations in MS brains are associated with altered distribution of tissue water (edema), this technique is highly useful for visualization of the

spatial distribution of lesions. Contrast in T_1 images is determined mainly by different lattice densities. Dense structures, such as compact white matter, have low T_1 values, whereas relatively loose structures, such as grey matter or lesions, have higher T_1 values.

To distinguish inflammatory active from inactive lesions, the paramagnetic dye gadolinium-DTPA is intravenously injected ($0.1\text{--}0.3\text{ mmol kg}^{-1}$) and, in areas of increased blood-brain barrier permeability, leaks into the brain parenchyma, causing local enhancement of the T_1 -weighted signal intensity.

A third important MRI technique in MS is magnetization transfer ratio (MTR) imaging. The MTR of a given tissue is defined as the ratio of free protons versus protons bound to tissue macromolecules.

For further information, see <http://www.cis.rit.edu/htbooks/mri>.

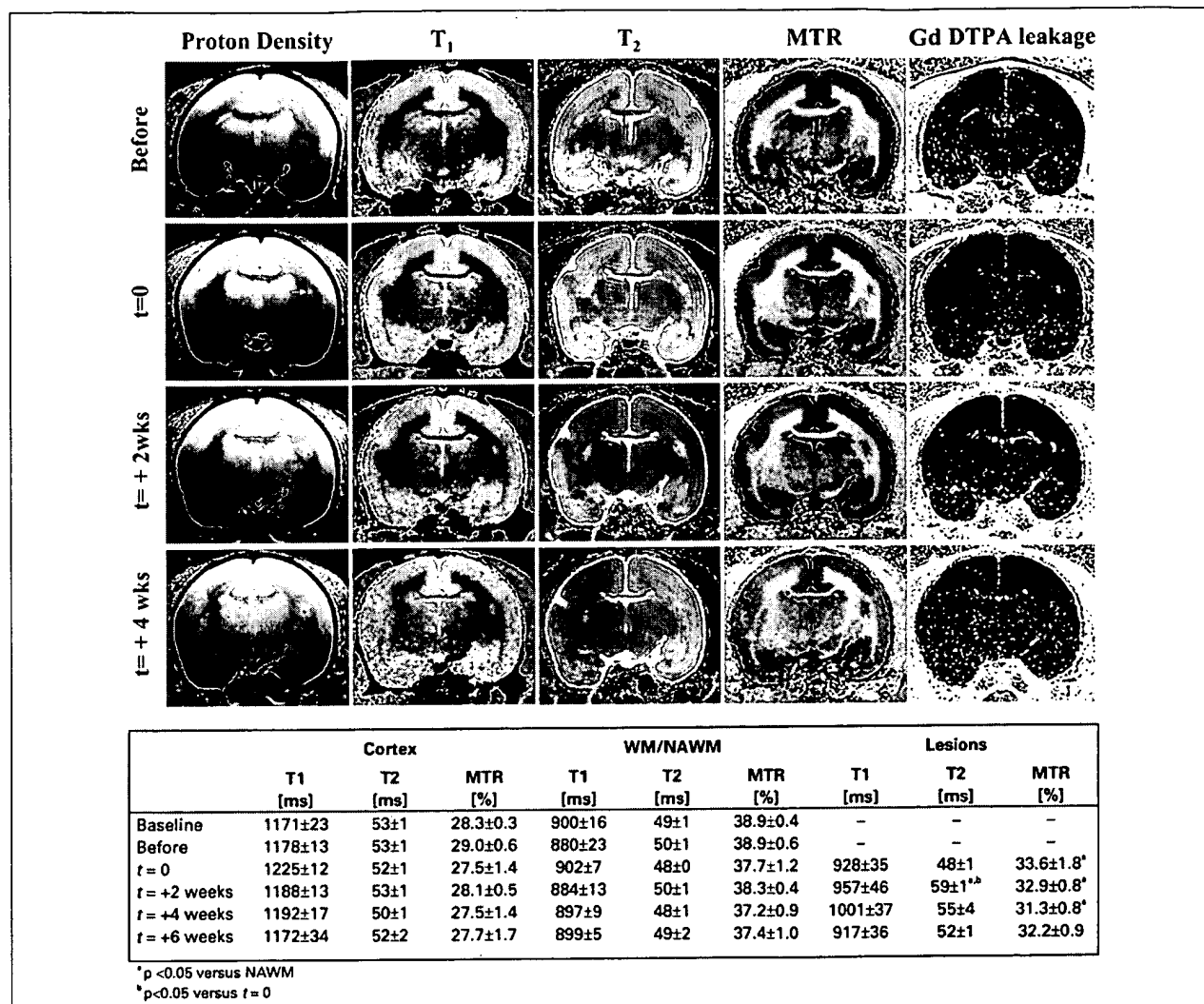


Figure 2. Serial magnetic resonance (MR) imaging of a marmoset brain affected by recombinant human myelin/oligodendrocyte glycoprotein (rhMOG)-induced experimental autoimmune encephalomyelitis (EAE). Images were recorded at ~ 14 day intervals. Depicted are: in column 1, proton density images, which are used for the sensitive detection of lesions; in columns 2 and 3, T₁ and T₂ relaxation time images; in column 4, MTR images, and; in column 5, images created by subtracting images recorded before and after the intravenous injection of gadolinium-DTPA (Gd-DTPA) as a probe for the detection of blood-brain barrier leakage. The arrow in the proton density image points to a lesion, which, in retrospect, could already be detected two weeks earlier as a focal leakage of the blood-brain barrier. The table shows the progression of quantitative MR parameters during the progression of EAE of cortex, white matter (WM), normal appearing white matter (NAWM) and lesions in brains from rhMOG-immunized marmosets (n = 5; — indicates no data).

Chemometric analysis of urines from EAE-affected marmosets

Most imaging techniques (X-ray, PET and MRI) are expensive and can cause discomfort to patients; therefore, they are not usually performed on a frequent basis. Hence, there is increasing interest in the identification in body fluids of biomarkers of pathological processes [46]. Biomarkers in urine are an ideal tool for non-invasive diagnosis of disease in monkeys. For example, the urinary excretion rates of hydroxylslyl- and lysyl-pyrridinolines (HP and LP) reflect the inflammatory degradation of joint collagen in arthritic rhesus monkeys [47]. This technique is of special diagnostic interest for the early stages of arthritis, when joint destruction is not macroscopically visible and is difficult to assess from X-rays. Excreted HP

and LP have also been very useful non-measurements for the effectiveness of new therapies for joint protection [48].

Biomarkers for inflammation (neopterin and cytokines) [49,50] and axonal destruction (N-acetylaspartate) have been detected in the urine of patients with MS. However, proposed biomarkers for demyelination, such as MBP fragments or cresol-sulphate [51,52] have proved unreliable. ¹HNMR spectroscopy, combined with pattern recognition-based techniques, has been used to investigate whether disease-associated changes can be detected in the urine of patients with MS and EAE-affected marmosets (Box 2). The chemometric analysis explained in Box 2 revealed marked characteristic differences between urine from patients with MS and relevant controls (i.e. urine from patients with other neurological diseases or from

Box 2. Proton-NMR spectroscopy and pattern recognition**NMR spectroscopy**

High-resolution proton nuclear magnetic resonance (^1H NMR) spectroscopy is an analytical technique that enables the simultaneous detection of a broad array of chemical entities in solutions of unknown composition. Aqueous solutions, such as urine, must be preprocessed before they can be analyzed. Because differences in pH between biological samples can affect the outcome of the ^1H NMR analysis, samples have to be buffered, for example, by adding an equal volume of phosphate-buffered saline (pH 7.4). To circumvent the dominant H_2O peak, samples are lyophilized and dissolved in D_2O . Sodium-trimethylsilyl- [2,2,3,3,2H₄]-1-propionate is added as an internal NMR reference. When measured using a spectrometer, peaks attributable to dietary components, such as glucose, saccharose, fructose, or agents involved in long term storage of the samples, such as hippuric acid and benzoic acid, are removed from the spectra before data processing. For normalization of spectra from urine samples collected at different time points and from different monkeys, all intensities are normalized against the -CH₃ peak of creatinine.

Data processing

Figure 1b shows two urine spectra from the same monkey; sample A was collected before disease induction and sample F at the height of clinical experimental autoimmune encephalomyelitis (Figure 1a: graph shows disease progression of three individual monkeys; 9338, 9314 and 9335).

It is difficult to see differences between two spectra visually. A ^1H NMR spectrum of one urine sample represents a set of several hundred data points. It is virtually impossible to detect systematic differences between larger groups of spectra without the use of pattern recognition techniques. In Figure 1b, small differences between the NMR positions of the same points in different spectra were first corrected with a partial linear fit algorithm, with which spectra can be aligned [54]. Differences between these groups of spectra were analyzed by multivariate analysis; more specific principal component (PCA) in combination with discriminant (DA) analysis. When samples can be *a priori* defined as belonging to one cluster, DA is used to test the degree of similarity between clusters of samples [54–56].

Score plots are created to visualize the degree of similarity between clusters of samples in a two-dimensional plane determined by the two main discriminant axes (Figure 1c). Each cluster (A–F) corresponds to the samples from Figure 1a. Clusters overlap when the degree of similarity between the samples within the cluster is high. When clusters are clearly separated, it can be concluded that a significant difference exists between the constituents of the clusters. The score plot shows localization of the pre-disease clusters (A and B) in one quadrant, clearly separated from the post-disease samples. The variation between samples collected at one time point is low.

Graphical rotation of the discriminant axes in a score plot in the direction of a certain cluster separation generates a factor plot (Figure 1d). The resulting factor spectrum shows the most important

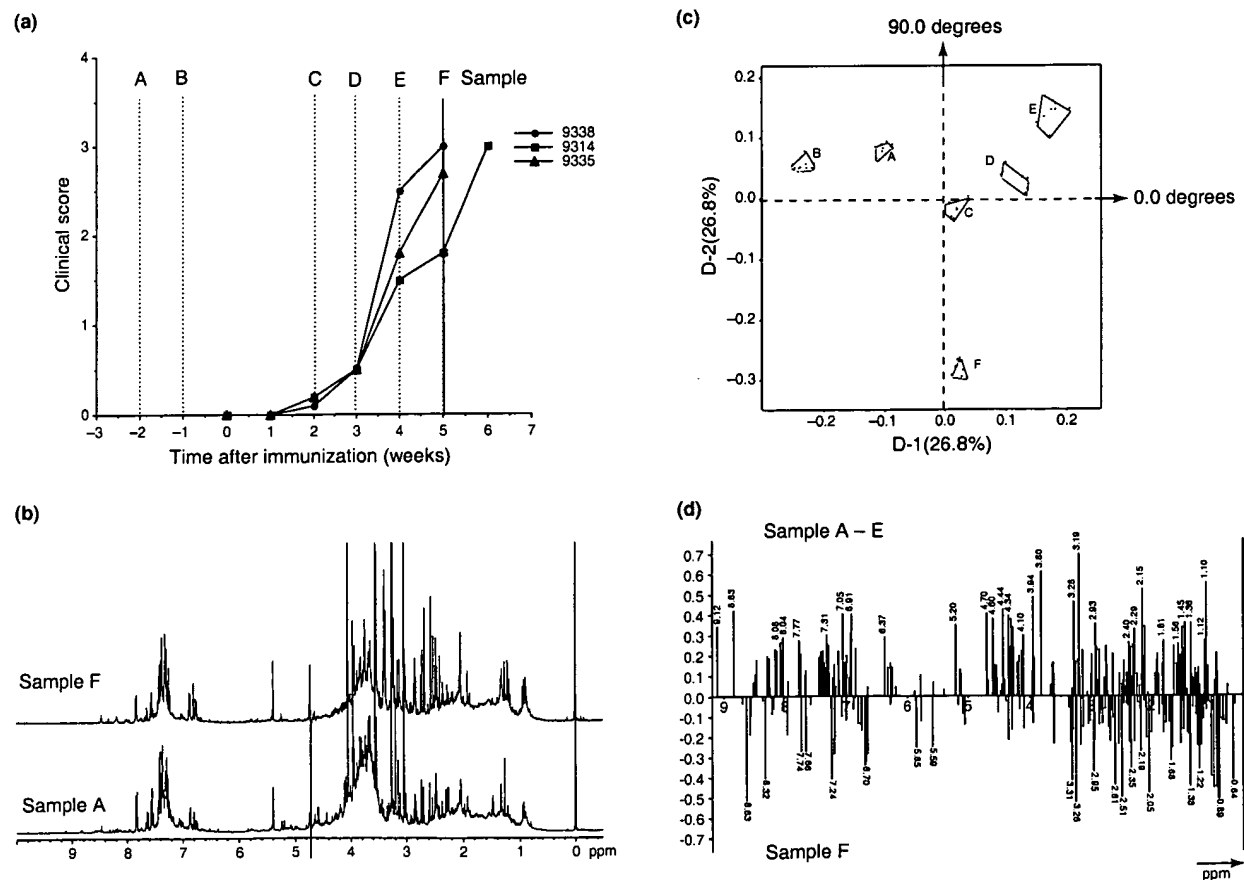


Figure 1.

NMR peaks contributing to the separation of clusters. Data from the factor plots are compared with a spectroscopy library to obtain tentative assignments of peaks to molecular structures. In this pseudo spectrum, the peaks in the positive direction correspond to compounds that predominate in samples A to E; peaks in the negative direction correspond to compounds predominating in samples F. The spectra from samples in cluster F shows relatively more (than average for the

whole set) compounds in the 0.5–3.5 part of the spectrum. Peaks of known urine biomarkers of MS have been tentatively identified, namely choline, inositol, neopterin and N-acetylaspartate. These figures have been reprinted with permission from reference [53].

This novel research approach, integrating high tech uroscopy techniques with pathology research, is known as metabonomics. For further information see references [57–59].

healthy volunteers [53]). However, the variability among patients in disease phenotype, medication and lifestyle complicates the identification of the markers that cause the spectral differences among urine samples. The marmoset EAE model offers the possibility to investigate this in a controlled setting; urine can be collected at defined time points before and after disease induction, and both medication and diet can be standardized.

In one study, urine was collected at weekly intervals from three common marmosets, in which EAE was induced by immunization with myelin from patients with MS (Box 2). Score plots revealed a marked change in the chemical composition associated with disease progression, such as neopterin, choline and inositol. ¹HNMR spectrum analysis of the urine from common marmosets with rhMOG-induced EAE also revealed a marked change in the aliphatic region of the spectrum [53]. Although promising, the development of a diagnostic test awaits elucidation of the exact chemical nature of NMR peaks in other regions of the spectrum.

Concluding remarks

We believe that the common marmoset is a useful tool for molecular research into MS. The outbred character of the (chronic) EAE model, together with the close genetic and immunological proximity of marmosets and humans, offer a unique experimental setting for the research of the immunopathogenic mechanisms in MS. EAE models in rodents have limited value as preclinical test systems for new therapies, because of the poor immunological cross-reactivity with humans. Moreover, the rat and mouse brain contains only a small amount of white matter, and therefore less useful for the imaging of CNS white matter pathology than primates. The neuropathological similarity of the marmoset EAE model with the prevalent type II lesion pathology in chronic MS has been well established [5,24]. Our ability to examine lesion pathology using non-invasive brain imaging techniques and chemometric urine analysis provides a novel opportunity for preclinical therapy development for neurological disorders.

References

- Compston, A. and Coles, A. (2002) Multiple sclerosis. *Lancet* 359, 1221–1231
- Lassmann, H. (1998) Neuropathology in multiple sclerosis: new concepts. *Mult. Scler.* 4, 93–98
- Lucchinetti, C. *et al.* (2000) Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann. Neurol.* 47, 707–717
- Trapp, B.D. *et al.* (1998) Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med.* 338, 278–285
- Brok, H.P. *et al.* (2001) Non-human primate models of multiple sclerosis. *Immunol. Rev.* 183, 173–185
- Polman, C.H. and Uitdehaag, B.M. (2003) New and emerging treatment options for multiple sclerosis. *Lancet Neurol.* 2, 563–566
- Bielekova, B. *et al.* (2000) Encephalitogenic potential of the myelin basic protein peptide (amino acids 83–99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat. Med.* 6, 1167–1175
- van Oosten, B.W. *et al.* (1996) Increased MRI activity and immune activation in two multiple sclerosis patients treated with the monoclonal anti-tumor necrosis factor antibody cA2. *Neurology* 47, 1531–1534
- Contag, P.R. (2002) Whole-animal cellular and molecular imaging to accelerate drug development. *Drug Discov. Today* 7, 555–562
- Sachs, D.H. (2003) Tolerance: of mice and men. *J. Clin. Invest.* 111, 1819–1821
- 't Hart, B.A. *et al.* (2000) A new primate model for multiple sclerosis in the common marmoset. *Immunol. Today* 21, 290–297
- 't Hart, B.A. and Amor, S. (2003) The use of animal models to investigate the pathogenesis of neuroinflammatory disorders of the central nervous system. *Curr. Opin. Neurol.* 16, 375–383
- Adams, A.B. *et al.* (2003) Heterologous immunity provides a potent barrier to transplantation tolerance. *J. Clin. Invest.* 111, 1887–1895
- 't Hart, B.A. *et al.* (2003) Gene therapy in nonhuman primate models of human autoimmune disease. *Gene Ther.* 10, 890–901
- Marshall, J.W. and Ridley, R.M. (2003) Assessment of cognitive and motor deficits in a marmoset model of stroke. *ILAR J.* 44, 153–160
- Jenner, P. and Marsden, C.D. (1986) The actions of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in animals as a model of Parkinson's disease. *J. Neural Transm. Suppl.* 20, 11–39
- Baker, H.F. *et al.* (1994) Induction of beta (A4)-amyloid in primates by injection of Alzheimer's disease brain homogenate. Comparison with transmission of spongiform encephalopathy. *Mol. Neurobiol.* 8, 25–39
- Genain, C.P. and Hauser, S.L. (1997) Creation of a model for multiple sclerosis in *Callithrix jacchus* marmosets. *J. Mol. Med.* 75, 187–197
- Stephan, H. *et al.* (1980) *The Brain of the Common Marmoset (Callithrix jacchus)*. A Stereotactic Atlas, Springer Verlag
- Pryce, C., *et al.* eds (1997) *Marmosets and tamarins in biological and biomedical research* DSSD Imagery
- Haig, D. (1999) What is a marmoset? *Am. J. Primatol.* 49, 285–296
- Massacesi, L. *et al.* (1995) Active and passively induced experimental autoimmune encephalomyelitis in common marmosets: a new model for multiple sclerosis. *Ann. Neurol.* 37, 519–530
- Genain, C.P. *et al.* (1995) Antibody facilitation of multiple sclerosis-like lesions in a nonhuman primate. *J. Clin. Invest.* 96, 2966–2974
- Genain, C.P. and Hauser, S.L. (2001) Experimental allergic encephalomyelitis in the New World monkey *Callithrix jacchus*. *Immunol. Rev.* 183, 159–172
- Brok, H.P. *et al.* (2000) Myelin/oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis in common marmosets: the encephalitogenic T cell epitope pMOG24–36 is presented by a monomorphic MHC class II molecule. *J. Immunol.* 165, 1093–1101
- Antunes, S.G. *et al.* (1998) The common marmoset: a new world primate species with limited Mhc class II variability. *Proc. Natl. Acad. Sci. U. S. A.* 95, 11745–11750
- Bontrop, R.E. *et al.* (1999) Major histocompatibility complex class II polymorphisms in primates. *Immunol. Rev.* 167, 339–350
- 't Hart, B.A. *et al.* (1998) Histopathological characterization of magnetic resonance imaging- detectable brain white matter lesions in a primate model of multiple sclerosis: a correlative study in the experimental autoimmune encephalomyelitis model in common marmosets (*Callithrix jacchus*). *Am. J. Pathol.* 153, 649–663
- Mancardi, G. *et al.* (2001) Demyelination and axonal damage in a non-human primate model of multiple sclerosis. *J. Neurol. Sci.* 184, 41–49
- Raine, C.S. *et al.* (1999) Demyelination in primate autoimmune encephalomyelitis and acute multiple sclerosis lesions: a case for antigen-specific antibody mediation. *Ann. Neurol.* 46, 144–160

- 31 Laman, J.D. *et al.* (1998) Expression of accessory molecules and cytokines in acute EAE in marmoset monkeys (*Callithrix jacchus*). *J. Neuroimmunol.* 86, 30–45
- 32 Brok, H.P. *et al.* (2001) An extensive monoclonal antibody panel for the phenotyping of leukocyte subsets in the common marmoset and the cotton-top tamarin. *Cytometry* 45, 294–303
- 33 von Buedingen, H.C. *et al.* (2001) Characterization of the expressed immunoglobulin IGHV repertoire in the New World marmoset *Callithrix jacchus*. *Immunogenetics* 53, 557–563
- 34 de Vos, A.F. *et al.* (2002) Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs. *J. Immunol.* 169, 5415–5423
- 35 Genain, C.P. *et al.* (1999) Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nat. Med.* 5, 170–175
- 36 McFarland, H.I. *et al.* (1999) Determinant spreading associated with demyelination in a nonhuman primate model of multiple sclerosis. *J. Immunol.* 162, 2384–2390
- 37 't Hart, B.A. *et al.* (2001) The major histocompatibility complex influences the ethiopathogenesis of MS-like disease in primates at multiple levels. *Hum. Immunol.* 62, 1371–1381
- 38 Yu, M. *et al.* (1998) HLA-DP: a class II restriction molecule involved in epitope spreading during the development of multiple sclerosis. *Hum. Immunol.* 59, 15–24
- 39 Barkhof, F. and Scheltens, P. (2002) Imaging of white matter lesions. *Cerebrovasc. Dis.* 13 (Suppl 2), 21–30
- 40 Jordan, E.K. *et al.* (1999) Serial MR imaging of experimental autoimmune encephalomyelitis induced by human white matter or by chimeric myelin-basic and proteolipid protein in the common marmoset. *Am. J. Neuroradiol.* 20, 965–976
- 41 Brück, W. *et al.* (1995) Monocyte/macrophage differentiation in early multiple sclerosis lesions. *Ann. Neurol.* 38, 788–796
- 42 Bruck, W. *et al.* (1997) Inflammatory central nervous system demyelination: correlation of magnetic resonance imaging findings with lesion pathology. *Ann. Neurol.* 42, 783–793
- 43 Brok, H.P. *et al.* (2002) Prevention of experimental autoimmune encephalomyelitis in common marmosets using an anti-IL-12p40 monoclonal antibody. *J. Immunol.* 169, 6554–6563
- 44 Boon, L. *et al.* (2001) Prevention of experimental autoimmune encephalomyelitis in the common marmoset (*Callithrix jacchus*) using a chimeric antagonist monoclonal antibody against human CD40 is associated with altered B cell responses. *J. Immunol.* 167, 2942–2949
- 45 Laman, J.D. *et al.* (2002) Protection of marmoset monkeys against EAE by treatment with a murine antibody blocking CD40 (mu5D12). *Eur. J. Immunol.* 32, 2218–2228
- 46 Nicholson, J.K. *et al.* (2002) Metabonomics: a platform for studying drug toxicity and gene function. *Nat. Rev. Drug Discov.* 1, 153–161
- 47 't Hart, B.A. *et al.* (1998) Collagen-induced arthritis in rhesus monkeys: evaluation of markers for inflammation and joint degradation. *Br. J. Rheumatol.* 37, 314–323
- 48 Brok, H.P. *et al.* (2001) Prophylactic and therapeutic effects of a humanized monoclonal antibody against the IL-2 receptor (DACLI-ZUMAB) on collagen-induced arthritis (CIA) in rhesus monkeys. *Clin. Exp. Immunol.* 124, 134–141
- 49 Giovannoni, G. *et al.* (2000) Immunological time-course of gadolinium-enhancing MRI lesions in patients with multiple sclerosis. *Eur. Neurol.* 44, 222–228
- 50 Sorensen, P.S. (1999) Biological markers in body fluids for activity and progression in multiple sclerosis. *Mult. Scler.* 5, 287–290
- 51 Whitaker, J.N. (1998) Myelin basic protein-like material in the urine of multiple sclerosis patients: relationships to clinical and neuroimaging changes. *Mult. Scler.* 4, 243–246
- 52 Cao, L. *et al.* (2000) p-Cresol sulfate is the dominant component of urinary myelin basic protein like material. *Arch. Biochem. Biophys.* 377, 9–21
- 53 't Hart, B.A. *et al.* (2003) 1H-NMR spectroscopy combined with pattern recognition analysis reveals characteristic chemical patterns in urines of MS patients and non-human primates with MS-like disease. *J. Neurol. Sci.* 212, 21–30
- 54 Vogels, J.T.W.E. *et al.* (1996) Partial linear fit: a new NMR spectroscopy pre-processing tool for pattern recognition applications. *J. Chemometrics* 10, 425–438
- 55 Hoogerbrugge, R. *et al.* (1983) Discriminant analysis by double stage principal component analysis. *Anal. Chem.* 55, 1710–1712
- 56 Windig, W. *et al.* (1984) Nonsupervised numerical component extraction from pyrolysis mass spectra of complex mixtures. *Anal. Chem.* 56, 2297–2303
- 57 Nicholson, J.K. and Wilson, I.D. (1987) High resolution nuclear magnetic resonance spectroscopy of biological samples as an aid to drug development. *Prog. Drug Res.* 31, 427–479
- 58 Nicholson, J.K. *et al.* (1999) 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 29, 1181–1189
- 59 Nicholson, J.K. *et al.* (2002) Metabonomics: a platform for studying drug toxicity and gene function. *Nat. Rev. Drug Discov.* 1, 153–161

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Antibody Facilitation of Multiple Sclerosis-like Lesions in a Nonhuman Primate

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Abstract

In the human disease multiple sclerosis (MS), the immune mechanisms responsible for selective destruction of central nervous system myelin are unknown. In the common marmoset *Callithrix jacchus*, a unique demyelinating form of experimental allergic encephalomyelitis resembling MS can be induced by immunization with whole myelin. Here we show that the MS-like lesion can be reproduced by immunization against the extracellular domain of a single myelin protein, myelin/oligodendrocyte glycoprotein (MOG). By contrast, immunization against the quantitatively major myelin proteins myelin basic protein or proteolipid protein results in inflammation but little or no demyelination. Furthermore, in the presence of encephalitogenic (e.g., disease-inducing) T cells, the fully demyelinated lesion is reconstructed by systemic administration of IgG purified from whole myelin-, or MOG-immunized animals, and equally by a monoclonal antibody against MOG, but not by control IgG. Encephalitogenic T cells may contribute to the MS-like lesion through disruption of the blood brain barrier that permits access of demyelinating antibody into the nervous system. The identification of MOG as a major target antigen for autoimmune demyelination in a nonhuman primate should facilitate development of specific immunotherapies for human MS. (*J. Clin. Invest.* 1995. 96:2966–2974.) **Key words:** experimental allergic encephalomyelitis • common marmoset • myelin/oligodendrocyte glycoprotein • inflammation • demyelinating antibody

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1. Abbreviations used in this paper: CNS, central nervous system; CSF, cerebrospinal fluid; EAE, experimental allergic encephalomyelitis; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; MOG, myelin/oligodendrocyte glycoprotein; MS, multiple sclerosis; PLP, proteolipid apoprotein; rMOG, recombinant MOG; WM, white matter.

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Introduction

Multiple sclerosis (MS)¹ lesions are characterized by large, sharply demarcated areas of demyelination associated with macrophage infiltration, accumulations of perivascular and parenchymal T cells, and progressive astrocytic proliferation (gliosis) (1). Despite observations of cellular (2, 3) and humoral (4) immune reactivity to myelin autoantigens in some patients, and some similarity to the autoimmune disease model experimental allergic encephalomyelitis (EAE) (1), the etiology of MS remains unknown. In inbred rodents, antigens capable of inducing EAE by active immunization include the predominant myelin proteins myelin basic protein (MBP) and proteolipid apoprotein (PLP) (5, 6), the minor myelin protein myelin/oligodendrocyte glycoprotein (MOG) (7), and myelin lipids (8) or nonmyelin proteins of central nervous system (CNS) (9). Reconstitution and adoptive transfer experiments (10–12) have established a primary role for antigen-specific T cells in EAE. Antibody participation has also been suggested by observations that intact B cell function is required for full expression of EAE (13), and that clinical signs are enhanced and demyelination occurs following the administration of demyelinating antibodies. These may be directed against gangliosides and galactocerebroside (14), myelin associated glycoprotein (15), or MOG (7). Pathologically, typical acute EAE differs substantially from MS in that prominent inflammation occurs in gray, white and meningeal structures and demyelination is scant or absent.

A primary demyelinating form of EAE occurs in the common marmoset *C. jacchus* after immunization with whole white matter (WM). Typically, a chronic, relapsing remitting clinical course is observed. Pathologically, the disorder is characterized by the early development of large, sharply demarcated areas of demyelination and gliosis reminiscent of MS plaques (16). *C. jacchus* marmosets are also notable for the presence of permanent and stable bone marrow chimerism between fraternal siblings, resulting from sharing of a common fetal blood supply between developing embryos in utero. In contrast to rodent EAE (10–12), adoptive transfer of MBP-reactive T cells in this primate species was not sufficient to reproduce the distinctive pathologic lesion observed following active immunization with WM (17). Thus the pathogenesis of the demyelinating phenotype was unknown. The present study was undertaken to identify the target antigen(s) and immune mechanisms responsible for the MS-like lesion in *C. jacchus*.

Methods

Antigens. human MBP and human PLP were purified as previously described (18, 19). The nonglycosylated recombinant fusion protein

Table 1. Clinical and Pathologic Characteristics of *C. jacchus* EAE

Antigen	Animal	EAE (day of onset)	Duration (days) initial/relapse	Maximum grade*	CSF (WBC/mm ³)*	Pathology			Day of death
						Subpial inflammation	Perivascular cuffing	Demyelination (plaques)	
HWM 100 mg	140-90	17	7	4 (24)	220 (24)	+	+++	+++	24
	139-90	25	7	3 (32)	580 (32)	+	+++	+++	32
MBP 1 mg [†]	361-90	17	14/2	2 (51)	80 (51)	+	0	0	51
MBP 1 mg [†]	362-90	36	10/24	2 (80)	ND	0	0	0	80
MBP 500 µg	567-92	18	3	2 (20)	76 (20)	+	0	0	20
aa 1-21 [†]	36-91	—	—	0	ND		ND		
	37-91	—	—	0	ND	0	0	0	51
aa 82-103 [†]	100-89	36	10/27	1	ND		ND		
	407-89	25	33/—	1	ND		ND		
aa 153-174 [†]	296-90	18	29	2 (47)	ND	+	0	0	47
	297-90	17	30	1 (46)	ND	0	0	0	47
PLP 100 µg [†]	325-91	21	42/91	2 (51)	140 (151)	0	0	0	158
	190-91	24	30/14	2 (100)	60 (87)	0	0	0	100
PLP 200 µg	267-88	9	3	2 (12)	180 (12)	0	0	0	12
PLP 200 µg [†]	124-93	14	17/57	2 (28)	180 (103)	+	+	+	103
PLP 200 µg + MBP 250 µg	88-88	17	10	3 (27)	220 (27)	+	+	+	27
rMOG 100 µg	123-93	16	2	2 (16)	140 (16)	+	0	0	17
	126-93	17	2	3 (18)	360 (18)	+	+++	+++	18
	83-89	14	54	4 (68)	120 (16)	+	++	+++	68
PLP 200 µg + MBP 250 µg + rMOG 100 µg	109-90	17	9	3 (25)	420 (25)	+	+++	+++	25

* Number in parentheses refer to days after the first immunization. [†] Animals were rechallenged at days 46 after the first immunization. ND, not done.

MOG (rMOG) containing the extracellular domain of rat MOG (aa 1-125) was prepared as described in (20). The sequence of the construct is extended for the amino acids (Met)-Arg-Gly-Ser at its NH₂ terminus, and for Arg-Ser-Gln-Ser-(His) 6 at its COOH terminus. The purity of all antigen preparations was confirmed by SDS-PAGE/Coomassie blue staining. MBP, major 18.5-kD band; PLP, 24 and 20-kD bands; MOG, 14 and 28 kD aggregate form. 20-mer overlapping peptides of MBP were derived from the sequence of human MBP, which differs from monkey MBP by 4 of 172 aminoacids (5). The MOG overlapping peptides were synthesized according to the published sequence of rat MOG (21).

Induction of EAE. Animals were immunized with the indicated amounts of myelin proteins (Table 1) or 100 µg MBP-peptides dissolved in 0.25 ml phosphate-buffered saline and mixed with an equal volume of complete Freund's adjuvant supplemented with 3 mg/ml of H37 Ra killed mycobacterium. Three MBP peptides corresponding to regions of human MBP known to be encephalitogenic in *C. jacchus* (17) were tested (aa 1-21, aa 82-103, and aa 153-174). All animals received 10¹⁰ killed *Bordetella pertussis* organisms intravenously on the day of immunization and 48 h later. Animals were cared for in full accordance with institutional guidelines. Cerebrospinal fluid (CSF) was obtained by puncture of the cisterna magna. All procedures were performed under brief ketamine (10 mg/kg i.m.) anesthesia. Animals were killed under ketamine anesthesia with a lethal dose of intravenous pentobarbital. Histopathological examinations were performed on formalin-fixed tissues. EAE was scored on a clinical scale of 1 to 5 by observers blinded to the study, and neuropathologic findings of inflammation and demyelination were graded according to a scale of 0 to 3+ (16).

Immunocytochemistry. Antibodies used in this study: CD3, rabbit anti human pan T cell; CD20, monoclonal mouse anti human B cell; CD68, monoclonal anti human macrophage (all from Dako); glial fibrillary acidic protein (GFAP), clone G.A.5 (Boehringer Mannheim). The immunoperoxidase method with [3,3'-diamino-benzidine] (Bio-

genex) was used. Positive controls for marmoset tissues were obtained using formalin-fixed spleen and lymph node tissues from normal *C. jacchus*.

Immune reactivity to myelin antigens. T cell proliferative responses were measured using 2 × 10⁵ freshly isolated PBMC plated in 96-well plates in 200 µl of AIMV medium (GIBCO BRL, Gaithersburg, MD) with the following antigens: human WM (0.1%, wt/vol), MBP (50 µg/ml), PLP (10 µg/ml), or rMOG (10 µg/ml). After 48 h incubation, 0.5 µCi [³H]thymidine was added and the cells were harvested 18 h later. Stimulation indices were calculated as the ratios of ³H incorporation in stimulated/unstimulated (medium alone) PBMC. Antibody responses were tested in serial dilutions of *C. jacchus* sera using a dot-blot microfiltration apparatus with purified MBP (250 ng/dot), PLP (500 ng/dot), and rMOG (500 ng/dot) adsorbed on 0.45 µm nitrocellulose membranes. Color development was achieved using anti-monkey IgG coupled to immunoperoxidase (Sigma Chemical Co., St. Louis, MO) at a dilution of 1/4,000 and enhanced DAB substrate according to the manufacturer's instructions (Pierce, Rockford, IL). Fine specificity of anti-MOG antibodies were determined by ELISA in 96-well plates coated using 1 µg of synthetic rat MOG-peptides per well, with 100 µl of sera (1:200 dilution) and 100 µl of peroxidase-conjugated anti-monkey IgG (1:6,000; Sigma Chemical Co.). Plates were developed with [o-phenylenediamine]-peroxidase substrate and read at 490 nm.

Adoptive transfer of autoantibodies. Native *C. jacchus* antibodies (IgG) were purified from plasma by affinity chromatography over protein A-agarose using the MAPS buffer system (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions, and then dialyzed against phosphate buffered saline before injection. The murine monoclonal anti-MOG antibody 8.18.C5 was purified from ascites supernatants (22). Animals were immunized with native MBP (500 µg) and, at the time when clinical signs of EAE, CSF pleocytosis and T cell reactivity to MBP appeared, 150 mg/kg of *C. jacchus* IgG or 25 mg/kg of 8.18.C5 were administered by a single intravenous injection.

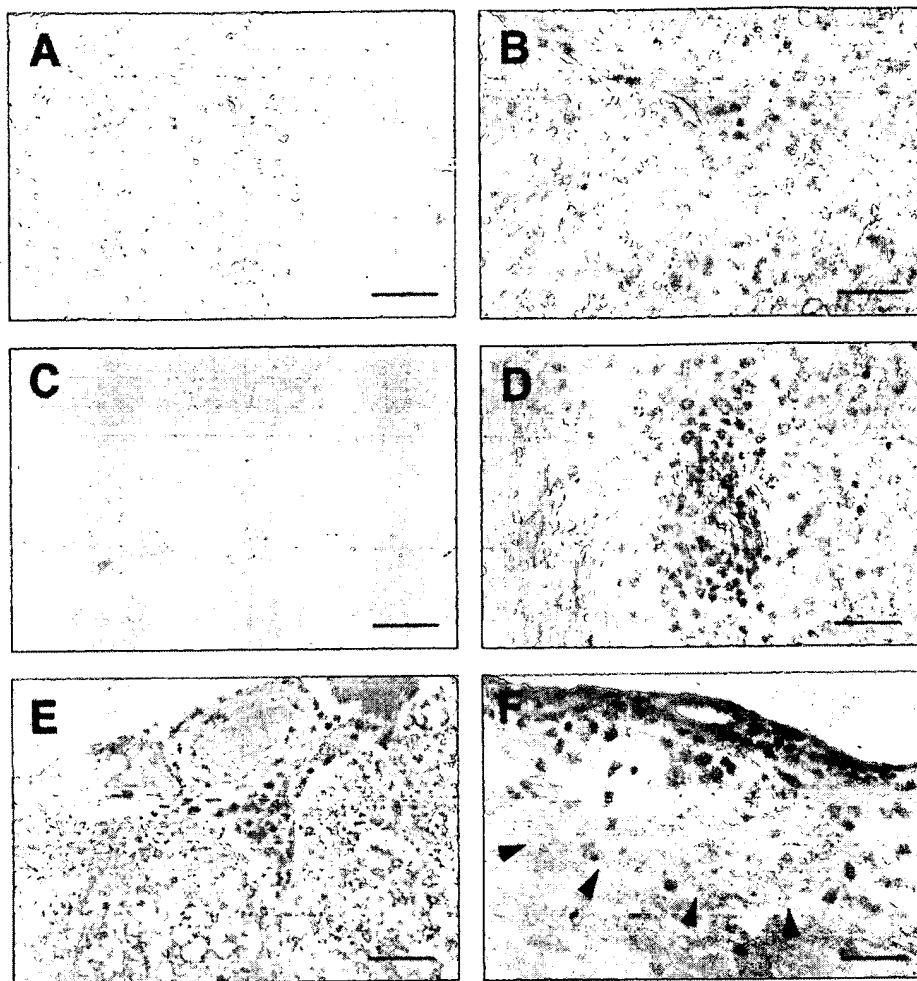


Figure 1. Representative pathologic findings of EAE in *C. jacchus*. Low (A, C, G, I, and K) and high (B, D, E, F, H, J, and L) power views of fields stained with hematoxylin/eosin (G) or luxol fast blue/periodic acid Schiff. Bars, 200 and 40 μ m, respectively. (A and B) Acute EAE induced by WM immunization. In A, foci of perivascular cuffing with pronounced demyelination. In B, higher magnification illustrates monocyte and macrophage infiltration; (C and D) EAE induced by rMOG. Note extensive perivascular cuffing with demyelination, and similar morphology of inflammatory cells as in WM-EAE; (E) EAE induced by MBP. Note subpial infiltration by mononuclear cells in spinal cord, but absence of parenchymal infiltration or demyelination; (F) in contrast, rMOG-induced EAE displayed prominent demyelination (arrowheads) underlying a subpial infiltrate in the spinal cord; (G) MBP-induced EAE. Low power view of a subpial infiltrate (hematoxylin/eosin); (H) MBP-induced EAE. A perivascular inflammatory infiltrate in a dorsal peripheral root;

(I) PLP-induced EAE illustrating a single inflammatory infiltrate (arrows) in the brain stem. (animal 124-93). The arrowhead indicates the area shown at higher power in J; (J) note the incomplete perivascular demyelination (highlighted by the arrowheads); (K and L) MBP + PLP-induced EAE. Cervical spinal cord section illustrating an infiltrate in the lateral funiculus (K, arrow), comprised of perivascular monocytes (L, arrow) with accompanying areas of incomplete demyelination.

Results

To identify the role of different myelin proteins in *C. jacchus* EAE, groups of marmosets were actively immunized with either human WM, native MBP, synthetic peptides of MBP, PLP, or rMOG, either alone or in combination. All animals developed clinical signs of EAE within 36 d of immunization, with the exception of two animals immunized with a peptide corresponding to the amino acid sequence 1–21 of MBP (Table I). Severe clinical EAE (grades 3 and 4) and CSF pleocytosis indicative of CNS inflammation occurred solely in animals immunized with whole WM, rMOG alone or rMOG in combination with PLP and MBP. Immunization with either MBP or PLP resulted in mild clinical signs (grade 2) and a mild degree of CSF pleocytosis; immunization with synthetic peptides corresponding amino acid sequences 82–103 or 153–176 of MBP also induced mild clinical disease, similar to that observed with whole MBP. After recovery from acute MBP or PLP induced

EAE, rechallenge with the same protein resulted in the development of new clinical signs but did not increase disease severity. Thus by clinical criteria, rMOG was a more potent autoantigen than either MBP or PLP in *C. jacchus*.

The characteristic pathology of EAE induced by immunization with whole WM was also reproduced by immunization with rMOG, but not with MBP or PLP. Two of three animals immunized with rMOG, and one animal immunized with rMOG in combination with MBP and PLP, developed intense CNS perivascular infiltration by mononuclear cells and macrophages accompanied by prominent concentric demyelination (Fig. 1). By contrast, with MBP immunization subpial inflammation was present but no demyelinating lesions could be identified. Inflammation in MBP-immunized animals was also present in spinal nerve roots (in particular the cauda equina), and it is possible that these inflammatory changes in the peripheral nervous system contributed to the clinical signs. These findings were identical to those previously reported to follow adoptive

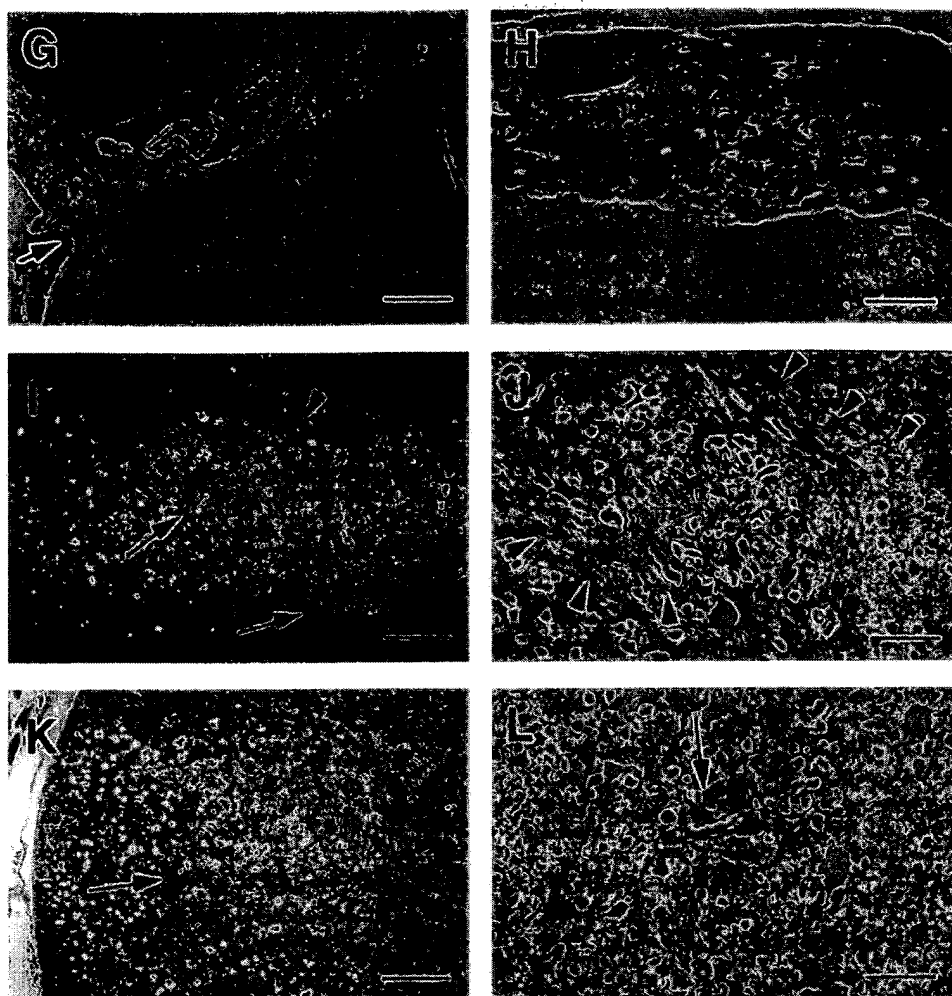


Figure 1 (Continued)

transfer of MBP-reactive T cell clones in this species (17). In PLP-immunized animals, subpial inflammation and occasional parenchymal perivascular mononuclear cell infiltrates occurred but demyelination was not present with the exception of a single focus in the brain stem of one of four PLP-immunized marmosets (Fig. 1, I–J). Immunization of another animal with a combination of PLP and MBP resulted in a more severe form of EAE than present with either antigen alone. In this animal, rare foci of perivascular cuffing, macrophage accumulation and incomplete demyelination were present in the spinal cord (Fig. 1, K–L). In both WM and rMOG EAE, acute inflammatory lesions were comprised predominantly of T cells located in perivascular cuffs and surrounding parenchyma (Fig. 2). B-cells were also present but in lower numbers than T cells. Marked astrocytic proliferation (gliosis) was also characteristic of both WM and rMOG EAE. Because no parenchymal inflammatory lesions were present in MBP-immunized animals, immunohistochemical studies in this model were confined to the characterization of the meningeal infiltrate. The meningitis in MBP-immunized animals consisted predominantly of CD3+ T cells (data not shown).

We examined circulating T cell and antibody responses in

all immunized *C. jacchus* at the time of acute clinical disease. T cell proliferative responses could be readily detected against both MBP and rMOG but, consistent with earlier data (16), in no animal could a response to PLP be identified (Fig. 3 A). Circulating antibodies against the immunizing protein developed in the MBP, PLP, and rMOG groups, and in WM-immunized animals antibodies against all three proteins were present (Fig. 3 B). PLP-immunized animals developed, in addition to anti-PLP antibodies, antibodies to both MBP and rMOG. Only whole WM and rMOG produced demyelinating forms of EAE (Table 1), thus the MS-like lesion appeared to require both a T cell response to either MBP or MOG, and an antibody response against MOG. Overlapping synthetic peptides were used to map the fine specificity of the antibody response to rMOG. In each of three animals examined, this response was restricted to two regions of the protein spanning residues 1–25 and 50–79 (Fig. 3 C).

To identify a role for pathogenic autoantibody in plaque formation, purified IgG antibodies were adoptively transferred to MBP-immunized recipients. As described above, immunization with MBP results in clinically mild EAE and no demyelination. IgG was prepared from the plasma of control animals

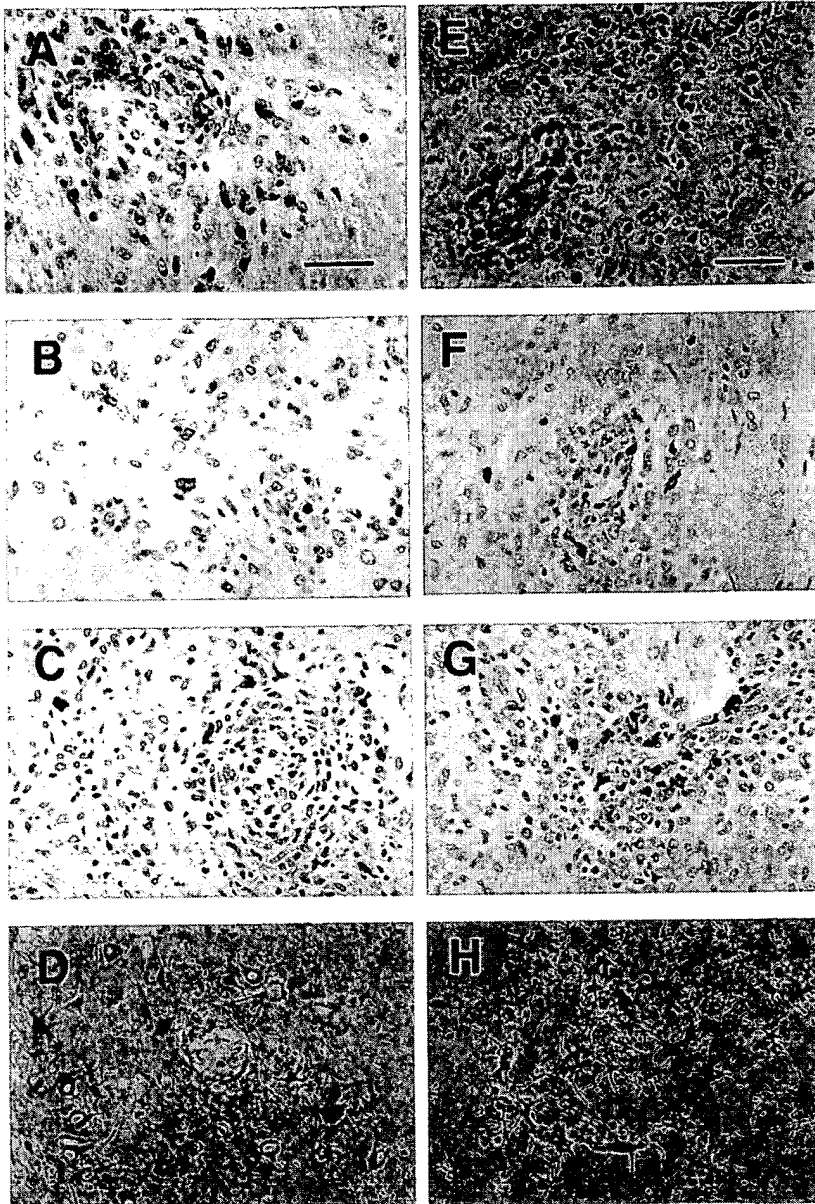


Figure 2. Immunocytochemistry of EAE lesions. (A–D) WM-EAE and (E–H) rMOG-EAE. (A and E) CD3+ T cells constitute the majority of the perivascular infiltrates; some T cells are noted to invade the parenchyma at a distance from the Virchow-Robin space. (B and F) CD20+ B cells are present both in the perivascular space and parenchyma. (C and G) CD68+ macrophages, visible in areas of demyelination. (D and H) GFAP reactivity, illustrating intense peri-lesional gliosis. Bars, 40 μm.

(IgG-C) and also from animals with demyelinating forms of EAE induced by active immunization with either WM (IgG-WM) or rMOG (IgG-rMOG). Adoptive transfer of IgG-WM, but not IgG-C, resulted in clinical deterioration within 48 h of antibody administration, and pronounced demyelination observed post-mortem (Table II). Clinical deterioration and demyelination also followed administration of either IgG-rMOG or the MOG-specific mouse monoclonal antibody 8.18.C5. Histologically, plaque formation in antibody recipients was indistinguishable from that resulting from active immunization with WM or rMOG (Fig. 4). No demyelination was present in marmosets immunized with complete Freund's adjuvant and Bordetella pertussis alone who then received monoclonal anti-MOG antibody, suggesting that demyelinating antibody requires an

activated encephalitogenic T cell response to effectively target CNS myelin.

Discussion

This study establishes a primary role for MOG in an autoimmune MS-like illness in a primate. Although EAE was initially described in monkeys, the clinical course in primates tended to be hyperacute and the pathologic lesions hemorrhagic and necrotic rather than demyelinating in nature (23). With additional manipulations, for example steroid or other treatment administered during the acute period, chronic disease with demyelinating characteristics has been reported in primates (24, 25), but the models have been cumbersome to use and not pathologically

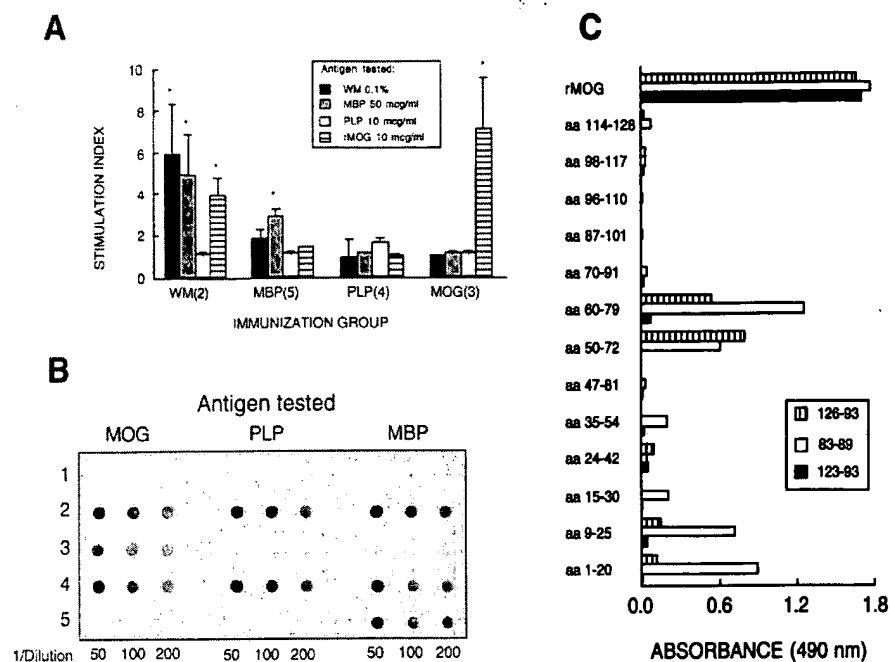


Figure 3. T cell and antibody responses to myelin antigens during *C. jacchus* EAE. (A) T cell proliferative responses against WM, MBP, PLP, and rMOG, measured as stimulation indices during acute EAE induced by different antigens. See Methods for details. For each immunization group the number of animals is shown in parentheses. * $P < 0.05$ compared with control (no antigen), Student's *t* test. (B) Antibody responses to MBP, PLP and rMOG in animals immunized with the different antigens. Representative data for each immunization group are illustrated as follows: row 1, control serum; row 2, WM; row 3, rMOG; row 4, PLP; row 5, MBP. (C) Fine specificity of anti-rMOG antibody responses in rMOG-immunized animals determined by ELISA. In individual animals, reactivity was assessed by the use of overlapping peptides corresponding to the extracellular domain of MOG. As shown in the

top lane, all three immunized animals displayed antibodies against the whole rMOG protein. Two regions of MOG, located between aa 1–25 and aa 50–79, could be identified as antigenic targets of the antibody response. See methods for details of the assay.

MS-like. The unique EAE-WM lesion in *C. jacchus*, characterized by widespread acute demyelination, demonstrated that an MS-like phenotype indeed could be created in a species phylogenetically close to humans. The different EAE phenotypes that occur in *C. jacchus* using various immunization or adoptive transfer strategies permitted the identification of the mechanisms responsible for the fully developed lesion to be defined. In addition to the role of MOG as an autoantigen, current data also demonstrate the importance of autoantibodies in plaque formation. Synergism between an encephalitogenic T cell response and specific demyelinating antibody appears to be required to produce the fully developed MS-like lesion. Both MBP and MOG appear to be effective T cell antigens in *C. jacchus*. Encephalitogenic T cells may function, at least in part,

through disruption of the blood brain barrier and facilitation of entry of autoantibody into the CNS.

The precise mechanisms that underlie immune-mediated demyelination in EAE (or in MS) are not known. It has been postulated that, following entry of sensitized T cells into the CNS, antigen recognition occurs in the context of antigen presentation by resident CNS cells (26). Inflammatory cytokines, including tumor necrosis factor- α and interferon- γ , are secreted and may directly injure oligodendrocytes, the myelin producing cells (27). Cytokine mediators are also likely to participate in recruitment of other inflammatory cells to the CNS for example macrophages, the cell type most often associated with demyelinating lesions. In EAE, a critical role for tumor necrosis factor- α in demyelination is suggested by the observation that rolipram, a

Table II. Adoptive Transfers of Autoantibodies in MBP-Immunized Marmosets

Animal	Onset of EAE (days)	CSF (WBC/mm ³)	Antibody (days pi)	EAE grade (before/after antibody)	Subpial inflammation	Pathology perivascular cuffing	Demyelination (plaques)	Day of death
120-92	13	45	IgG-WM (23)	2/3	+	++	++	37
90-88	21	70	IgG-WM (23)	2/3	+	++	++	25
91-88	13	32	8.18.C5 (23)	2/3	+	+	+	37
468-93	19	140	8.18.C5 (34)	2/3	+	++	++	36
499-93	9	NA	IgG-rMOG(32)	2/3	+	+++	++	41
102-89	26	52	rIgG-C (28)	2/2	+	0	0	30
119-92	41	NA	IgG-C (41)	2/2	+	0	0	43
102-93*	—	0	8.18.C5 (23)	0/0	0	0	0	25
506-93*	—	NA	8.18.C5 (23)	0/0	0	0	0	25

* Animals immunized with Freund's adjuvant and Bordetella pertussis only. NA, not available.

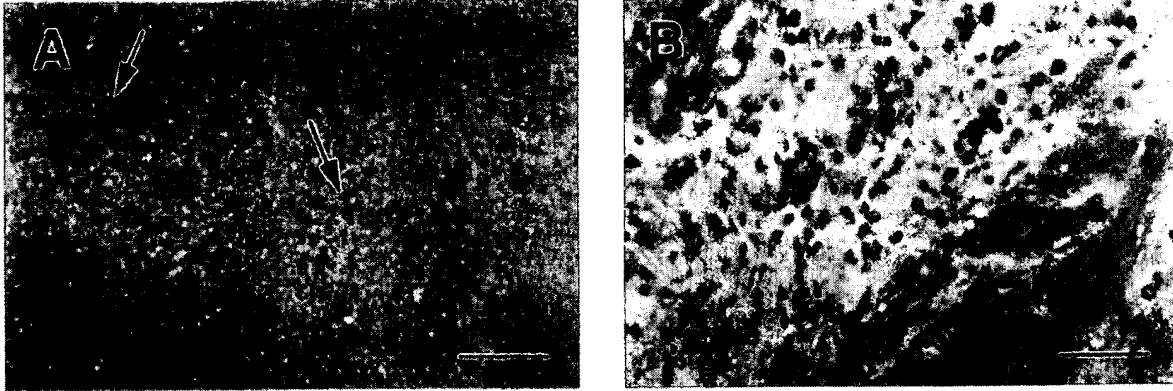


Figure 4. Adoptive transfer of demyelinating antibodies reproduces MS-like lesions in MBP-immunized *C. jacchus*. Typical lesions in the brain stem produced by intravenous administration of the monoclonal anti-MOG antibody 8.18.C5 (A) or IgG-WM (B), in animals previously immunized with MBP. Luxol fast blue/periodic acid Schiff. (A) Low power view of parenchymal infiltrates (arrows). Bar, 200 μ m. (B) High power view of an infiltrate, demonstrating that demyelination has occurred. Bar, 40 μ m.

selective inhibitor of the type IV phosphodiesterase, suppressed tumor necrosis factor α secretion, clinical disease manifestations, and demyelination (28, 29).

Earlier reports supported a role for antibody-mediated mechanisms in demyelination. In rodents, EAE can be converted from a nondemyelinating to a demyelinating form by administration of antimyelin antibodies. As in the current study, administration of demyelinating antibody was associated with a worsening of the clinical EAE score (7). Demyelinating activity of some autoantibodies correlated with their complement-fixing properties (30). In addition to direct complement-mediated tissue injury, antibodies might also facilitate demyelination via macrophage activation leading to cytokine-mediated injury to oligodendrocytes, myelin phagocytosis, or antibody dependent cell mediated cytotoxicity (31).

In previous experiments, MOG was identified as a potentially important encephalitogen in rodents. A relapsing remitting form of EAE can be induced by a synthetic peptide corresponding to amino acid residues 92–106 of MOG in SJL mice (20). In contrast to rodents, the *C. jacchus* marmoset appears to be exquisitely sensitive to immunization with MOG relative to the more abundant myelin proteins, MBP and PLP. The strong proliferative response to MOG observed in WM-immunized *C. jacchus* is noteworthy given its low abundance ($\sim 0.1\%$ of myelin proteins) in CNS. In the current study, the recombinant fusion protein rMOG was employed. This protein consists of the extracellular, immunoglobulin-like domain of rat MOG (21). Although the sequence of *C. jacchus* MOG is not known, the amino acid sequence of MOG is highly conserved between species. For example, human and rat MOG differ only by 12 amino acids within the immunoglobulin-like domain (21, 32). The importance of anti-MOG autoantibodies in demyelination might be due to the accessible localization of MOG, and in particular of the extracellular immunoglobulin-like domain of the molecule, at the outermost surface of the myelin sheath (33). In addition to anti-MOG IgG, other demyelinating autoantibodies may be present in IgG-WM that also contribute to the pathology in recipients of antibody transfers. Other potential autoantibodies notwithstanding, it is clear that anti-MOG IgG alone can facilitate demyelination in *C. jacchus*. In earlier in

vivo or in vitro studies, demyelination was found to result from antibodies that recognized gangliosides, galactocerebroside (14) or myelin associated glycoprotein (15). Characterization of the repertoire of antigens that are targeted by pathogenic demyelinating antibodies in *C. jacchus* is in progress.

In PLP-immunized marmosets, circulating antibodies not only to PLP but also to MBP and MOG were detected. The PLP preparation used for immunization contained no detectable immunoreactivity against either MBP or rMOG (Fig. 3 B, lines 3 and 5). Thus, it is unlikely that the PLP was contaminated with other myelin proteins. It is possible that the immune response to PLP resulted in production of antibody against one or several epitopes of PLP that were cross-reactive against other myelin proteins. More likely, antigenic spread due to secondary sensitization may explain the development of autoantibodies against MBP and MOG in PLP-immunized marmosets. This mechanism has been proposed to account for the expansion of antigenic spreading of encephalitogenic determinants during the course of disease in rodent models of EAE (34–36). It is of interest that little demyelination was present in PLP-immunized marmosets, despite the appearance of circulating antibodies reactive against rMOG. In these animals, there was no development of a proliferative T cell response against PLP, and with few exceptions perivascular cuffing in the CNS was lacking. Perhaps T cell activation in response to the dose of PLP used for immunization failed to disrupt the blood-brain barrier sufficiently to enhance passage of demyelinating antibody into the CNS. Alternatively, anti-MOG antibodies induced by PLP sensitization may be nonpathogenic. Some degree of demyelination was present in one marmoset immunized twice with PLP, and also in the animal immunized with a combination of MBP and PLP. This may indicate that PLP is effective at a higher dose, or that a synergism between MBP and PLP results in limited demyelination in *C. jacchus*.

Consistent with previous data (16) we did not detect a specific T cell response against PLP in either WM- or PLP-immunized animals. In preliminary experiments and in earlier experience (16), proliferation assays were performed utilizing concentrations of PLP ranging from 0.5 to 40 μ g/ml and in no case was a significant response detected. In addition, PBMC

from PLP-immunized animals did not proliferate against PLP-peptide 139–151 which represent a major encephalitogenic determinant of PLP in SJL/J mice (37). It is thus unlikely that a PLP-specific T cell response was present but not detected. In a different model of *C. jacchus* EAE in which a vaccinia-MBP construct was administered to modulate the disease course, proliferative responses to whole PLP were detected in some animals, indicating that the assay system was able to detect a response against this protein (C. Genain et al., manuscript in preparation). In the current study, high titers of anti-PLP antibodies were elicited in all PLP-immunized marmosets, raising the possibility that a Th2-like response to this protein occurs in this species.

Multiple lines of evidence support a role for antigen specific T cells reactive against MBP (2), PLP (38, 39) and MOG (40) in the pathogenesis of MS. A primary T cell etiology to MS has been hypothesized based upon these studies, by analogy to MBP-induced EAE in rodents (11, 12), by other reports of T cell abnormalities in MS patients (41), and by demonstration of genetic linkage of MS to the major histocompatibility complex (MHC) (42) and T cell receptor beta chain loci (43). In the CSF of individuals with MS, evidence of an antibody-mediated immune response includes findings of elevated levels of immunoglobulin (44), membrane attack complexes indicating complement-mediated injury (45), and autoantibodies to myelin proteins (4). Using various in vitro systems, demyelinating antibody activity has been reported (46), and also disputed (47), in MS. Current findings raise the possibility that both a T cell and an antibody response may be required for the fully developed MS lesion or plaque to occur. In MS, perivenous inflammation and enhanced vascular permeability is not infrequently present in the retina, a tissue devoid of myelin (48). This implies that cellular infiltration and blood-brain barrier breakdown in MS does not result only from the effects of T cells that recognize antigens unique to myelin. By analogy to *C. jacchus*, the demyelinating phenotype in MS could result from autoantibodies directed against accessible myelin determinants in the context of a T cell response that enhances antibody access to the target tissue. Further definition of the role of autoantibodies directed against MOG, a minor but highly encephalitogenic protein, in plaque formation will be of considerable interest.

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References

1. Raine, C. S. 1983. Multiple sclerosis and chronic relapsing EAE: comparative ultrastructural neuropathology. In *Multiple Sclerosis. Pathology, Diagnosis and Management*. J. F. Hallpike, G. W. M. Adams, and W. W. Tourtellote, editors. William and Wilkins Co., Baltimore. 413–460.
2. Allegretta, M., J. A. Nicklas, S. Sriram, and R. J. Albertini. 1990. T cells responsive to myelin basic protein in patients with multiple sclerosis. *Science (Wash. DC)*. 247:718–721.
3. Oksenberg, J. R., M. A. Panzara, A. B. Begovitch, D. Mitchell, H. A. Erlich, R. S. Murray, R. Shimonkevitz, M. Sherrity, J. Rothbard, C. C. A. Bernard, and L. Steinman. 1993. Selection for T cell receptor V β -D β -J β gene arrangements with specificity for a myelin basic protein peptide in brain lesions of multiple sclerosis. *Nature (Lond.)*. 362:68–70.
4. Panitch, H. S., C. J. Hooper, and K. P. Johnson. 1980. CSF antibody to myelin basic protein. Measurement in patients with multiple sclerosis and subacute sclerosing panencephalitis. *Arch. Neurol.* 37:206–209.
5. Tabira, T., and J. I. Kira. 1992. Strain and species differences of encephalitogenic determinants of myelin basic protein and proteolipid apoprotein. In *Myelin: Biology and Chemistry*. R. E. Martenson, editor. CRC Press, Inc., Boca Raton, FL. 783–799.
6. Tuohy, V. K. 1994. Peptide determinants of myelin proteolipid protein (PLP) in autoimmune demyelinating disease: a review. *Neurochem. Res.* 19:935–944.
7. Schluesener, H. J., R. A. Sobel, C. Linington, and H. L. Weiner. 1987. A monoclonal antibody against a myelin oligodendrocyte glycoprotein induces relapses and demyelination in central nervous system autoimmune disease. *J. Immunol.* 139:4016–4021.
8. Moore, G. R. W., U. Traugott, M. Farooq, W. T. Norton, and C. S. Raine. 1984. Experimental autoimmune encephalomyelitis. Augmentation of demyelination by different myelin lipids. *Lab. Invest.* 51:416–424.
9. Kojima, K., T. Berger, H. Lassmann, D. Hinze-Selch, Y. Zhang, J. Gehrmann, K. Reske, H. Wekerle, and C. Linington. 1994. Experimental autoimmune panencephalitis and uveoretinitis transferred to the Lewis rat by T lymphocytes specific for the S100 β molecule, a calcium binding protein of astroglia. *J. Exp. Med.* 180:817–829.
10. Paterson, P. Y. 1960. Transfer of allergic encephalomyelitis in rats by means of lymph node cells. *J. Exp. Med.* 111:119–135.
11. Pettinelli, C. B., and D. E. McFarlin. 1980. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1 + 2 – T lymphocytes. *J. Immunol.* 127:1420–1423.
12. Zamvil, S. S., D. J. Mitchell, A. C. Moore, K. Kitamura, L. Steinman, and J. B. Rothbard. 1986. T-cell epitope of the autoantigen myelin basic protein that induces encephalomyelitis. *Nature (Lond.)*. 324:258–260.
13. Gausas, J., P. Y. Paterson, A. D. Day, and M. C. Dal Canto. 1992. Intact B-cell activity is essential for complete expression of experimental allergic encephalomyelitis in Lewis rats. *Cell. Immunol.* 72:360–366.
14. Roth, G. A., M. Roytta, R. K. Yu, C. S. Raine, and M. B. Bornstein. 1985. Antisera to different glycolipids induce myelin alterations in mouse spinal cord tissue cultures. *Brain Res.* 339:9–18.
15. Sergott, R. C., M. J. Brown, R. P. Lisak, and S. L. Miller. 1988. Antibody to myelin-associated glycoprotein produces central nervous system demyelination. *Neurology*. 38:422–426.
16. Massacesi, L., C. P. Genain, D. Lee-Parritz, N. L. Letvin, D. Canfield, and S. L. Hauser. 1995. Active and passively induced experimental autoimmune encephalomyelitis in common marmosets: a new model for multiple sclerosis. *Ann. Neurol.* 37:519–530.
17. Genain, C. P., D. Lee-Parritz, M. H. Nguyen, L. Massacesi, N. Joshi, R. Ferrante, K. Hoffman, M. Moseley, N. L. Letvin, and S. L. Hauser. 1994. In healthy primates, circulating autoreactive T cells mediate autoimmune disease. *J. Clin. Invest.* 94:1339–1345.
18. Deibler, G. E., R. E. Martenson, and M. W. Kies. 1972. Large scale preparation of myelin basic protein in central nervous tissue of several mammalian species. *Prep. Biochem.* 2:139–165.
19. Lees, M. B., and J. D. Sakura. 1979. Preparation of proteolipids. In *Research Methods in Neurochemistry*. N. Marks, and R. Rodnight, editors. Plenum Press, New York. 354–370.
20. Amor, S., N. Groome, C. Linington, M. M. Morris, K. Dornair, M. V. Gardinier, J. M. Mathieu, and D. Baker. 1994. Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. *J. Immunol.* 153:4349–4356.
21. Gardinier, M. V., P. Amiguet, C. Linington, and J. M. Mathieu. 1992. Myelin/oligodendrocyte glycoprotein is a unique member of the immunoglobulin superfamily. *J. Neurosci. Res.* 33:177–187.
22. Linington, C., B. Engelhardt, G. Kapocs, and H. Lassmann. 1992. Induction of persistently demyelinated lesions in the rat following the repeated adoptive transfer of encephalitogenic T cells and demyelinating antibody. *J. Neuroimmunol.* 40:219–224.
23. Rivers, T. M., and F. F. Schwenker. 1935. Encephalomyelitis accompanied by myelin destruction experimentally produced in monkeys. *J. Exp. Med.* 61:689–702.
24. Shaw, C. M., E. C. Alvord, and S. Hornby. 1988. Chronic relapsing-relapsing experimental allergic encephalomyelitis induced in monkeys with homologous myelin basic protein. *Ann. Neurol.* 24:738–748.
25. Massacesi, L., N. Joshi, D. Lee-Parritz, A. Rombos, N. L. Letvin, and S. L. Hauser. 1992. Experimental allergic encephalomyelitis in cynomolgus monkeys: quantitation of T-cell response. *J. Clin. Invest.* 90:399–404.
26. Hickey, W. F., B. L. Hsu, and H. Kimura. 1991. T-lymphocyte entry into the central nervous system. *J. Neurosci. Res.* 28:254–260.

27. Benveniste, E. N. 1992. Inflammatory cytokines within the central nervous system: sources, function, and mechanism of action. *Am. J. Physiol.* 263:C1-C6.
28. Sommer, N., P.-A. Loschmann, G. H. Northoff, M. Weller, A. Steinbrecher, J. P. Steinbach, R. Lichtenfels, R. Meyermann, A. Riethmüller, A. Fontana, J. Dichgans, and R. Martin. 1995. The antidepressant rolipram suppresses cytokine production and prevents autoimmune encephalomyelitis. *Nature Med.* 1:244-248.
29. Genain, C. P., T. Roberts, R. L. Davis, M. H. Nguyen, A. Uccelli, D. Faulds, Y. Li, J. Hedgpeth, and S. L. Hauser. 1995. Prevention of autoimmune demyelination in non human primates by a cAMP-specific phosphodiesterase inhibitor. *Proc. Natl. Acad. Sci. USA.* 92:3601-3605.
30. Piddlesden, S. J., H. Lassmann, F. Zimprich, B. P. Morgan, and C. Linington. 1993. The demyelinating potential of antibodies to myelin oligodendrocyte glycoprotein is related to their ability to fix complement. *Am. J. Pathol.* 143:555-564.
31. Piddlesden, S., H. Lassmann, I. Laffañan, B. P. Morgan, and C. Linington. 1991. Antibody-mediated demyelination in experimental allergic encephalomyelitis is independent of complement membrane attack complex formation. *Clin. Exp. Immunol.* 83:245-250.
32. Pham-Dinh, D., B. Allinquant, M. Ruberg, B. Della-Gaspera, J. L. Nussbaum, and A. Dautigny. 1994. Characterization and expression of the cDNA coding for the human myelin/oligodendrocyte glycoprotein. *J. Neurochem.* 63:2353-2356.
33. Brunner, C., H. Lassmann, T. V. Wächter, J. M. Manthieu, and C. Linington. 1989. Differential ultrastructural localization of myelin basic protein, myelin/oligodendroglial glycoprotein, and 2',3'-cyclic nucleotide phosphodiesterase in the CNS of adult rats. *J. Neurochem.* 52:296-304.
34. McCarron, R. M., R. J. Fallis, and D. E. McFarlin. 1990. Alterations in T cell antigen specificity and class II restriction during the course of chronic relapsing experimental allergic encephalomyelitis. *J. Neuroimmunol.* 29:73-79.
35. Lehman, P. V., T. Forsthuber, A. Miller, and E. E. Sercarz. 1992. Spreading of T cell autoimmunity to cryptic determinants of an autoantigen. *Nature (Lond.)* 358:155-157.
36. Cross, A. H., V. K. Tuohy, and C. S. Raine. 1993. Development of reactivity to new myelin antigens during chronic relapsing autoimmune demyelination. *Cell. Immunol.* 146:261-269.
37. Tuohy, V. K., Z. Lu, R. A. Sobel, R. A. Laursen, and M. B. Lees. 1989. Identification of an encephalitogenic determinant of myelin proteolipid protein for SJL mice. *J. Immunol.* 142:1523-1527.
38. Zhang, J., S. Markovic-Plese, B. Lacet, J. Raus, H. L. Weiner, and D. A. Hafler. 1994. Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J. Exp. Med.* 179:973-984.
39. Pelfrey, C. M., J. L. Trotter, L. R. Tranquill, and H. F. McFarland. 1994. Identification of a second T cell epitope of human proteolipid protein (residues 89-106) recognized by proliferative and cytolytic CD4+ T cells from multiple sclerosis patients. *J. Neuroimmunol.* 53:153-161.
40. Kerlero de Rosbo, N., R. Milo, M. B. Lees, D. Burger, C. C. A. Bernard, and A. Ben-Nun. 1993. Reactivity to myelin antigens in multiple sclerosis. Peripheral blood lymphocytes respond predominantly to myelin oligodendrocyte glycoprotein. *J. Clin. Invest.* 92:2602-2608.
41. Hafler, D. A., M. Chofflon, E. Kurt-Jones, and H. L. Weiner. 1991. Interleukin-1 corrects the defective autologous mixed lymphocyte response in multiple sclerosis. *Clin. Immunol. Immunopathol.* 58:115-125.
42. Oksenberg, J. R., A. B. Begovitch, H. A. Erlich, and L. Steinman. 1993. Genetic factors in multiple sclerosis. *JAMA.* 270:2362-2369.
43. Seboun, E., M. A. Robinson, T. H. Doolittle, T. A. Ciulla, T. J. Kindt, and S. L. Hauser. 1989. A susceptibility locus for multiple sclerosis is linked to the T-cell receptor β chain complex. *Cell.* 57:1095-1100.
44. Tourtelotte, W. W., A. R. Potvin, J. O. Fleming, K. N. Murthy, J. Levy, K. Syndulco, and J. H. Potvin. 1980. Multiple sclerosis: measurement and validation of central nervous system IgG synthesis rate. *Neurology.* 30:240-244.
45. Scolding, N. J., B. P. Morgan, W. A. J. Houston, C. Linington, A. K. Campbell, and D. A. S. Compston. 1989. Vesicular removal by oligodendrocytes of membrane attack complexes formed by activated complement. *Nature (Lond.)* 339:620-622.
46. Bornstein, M. B., and S. H. Appel. 1965. Tissue culture studies in demyelination. *Ann. NY Acad. Sci.* 122:280-286.
47. Grundke-Iqbal, I., and M. B. Bornstein. 1980. Multiple sclerosis: serum gamma globulins and demyelination in organ cultures. *Neurology.* 30:749-754.
48. Vine, A. K. 1992. Severe periphrinitis, peripheral retinal ischemia, and preretinal neovascularization in patients with multiple sclerosis. *Am. J. Ophthalmol.* 113:28-32.

In Healthy Primates, Circulating Autoreactive T Cells Mediate Autoimmune Disease

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Abstract

A T cell response against myelin basic protein (MBP) is thought to contribute to the central nervous system (CNS) inflammation that occurs in the human demyelinating disease multiple sclerosis. To test whether MBP-reactive T cells that are normally retrieved from the circulation are capable of inducing CNS disease, MBP-reactive T cell clones were isolated from the peripheral blood of healthy, unimmunized *Callithrix jacchus* (*C. jacchus*) marmosets. This primate species is characterized by a natural chimerism of bone marrow elements between siblings that should make possible adoptive transfer of MBP-reactive T cells. We report that MBP-reactive T cell clones efficiently and reproducibly transfer CNS inflammatory disease between members of *C. jacchus* chimeric sets. The demyelination that is characteristic of experimental allergic encephalomyelitis induced in *C. jacchus* by immunization against human white matter did not occur after adoptive transfer of the MBP-reactive clones. It was noteworthy that encephalitogenic T cell clones were diverse in terms of their recognition of different epitopes of MBP, distinguishing the response in *C. jacchus* from that in some inbred rodents in which restricted recognition of MBP occurs. These findings are the first direct evidence that natural populations of circulating T cells directed against a CNS antigen can mediate an inflammatory autoimmune disease. (*J. Clin. Invest.* 1994. 94:1339–1345.) Key words: experimental allergic encephalomyelitis • multiple sclerosis • autoimmunity

Introduction

Myelin basic protein (MBP)¹ is, in most mammalian species, the major antigen responsible for experimental allergic encephalomyelitis (EAE), an autoimmune disease which serves as a model for the human demyelinating disease multiple sclerosis (MS).

In several rodent models, EAE is mediated by a T cell response to MBP, as demonstrated by the ability of MBP-reactive T cells derived from spleen and lymph nodes of an immunized donor to adoptively transfer disease into naive syngeneic recipients (1–4). In humans, MBP-reactive T cells are thought to play a role in acute disseminated encephalomyelitis that follows infection or vaccination (5, 6) and have long been suspected as effector cells in MS (7). MBP-reactive T cells are present in the circulation of patients with MS but are also present in healthy individuals (8–12). The role of these circulating T cell populations in the induction of human MS is unknown. Cross-species adoptive transfer experiments have been attempted with human donor cells transferred into rodent hosts, but have been unsuccessful (N. Joshi, unpublished observations) or have yielded uncertain results (13–15).

The common marmoset *Callithrix jacchus* (*C. jacchus*) represents a unique species for the study of T cell-mediated diseases in primates, because these monkeys are born as naturally occurring bone marrow chimeras. While the individual animals arise from separate ova that are fertilized independently, the placentas of the developing animals fuse, resulting in a crosscirculation of bone marrow-derived elements between the fetuses. Thus, while the animals are genetically distinct, they share, and are tolerant to, each other's bone marrow-derived cell populations (16). The natural chimerism in *C. jacchus* theoretically makes possible adoptive transfer of functional T cell populations between members of an outbred species without initiating an alloresponse. We have recently found that a relapsing–remitting form of EAE which bears a strong clinical and pathological resemblance to human MS can be induced in *C. jacchus* by immunization with human CNS white matter.² Immunized animals display specific proliferative responses to MBP, suggesting that MBP is an autoantigen in *C. jacchus* EAE. Thus, this outbred species of non-human primate is characterized by bone marrow chimerism and susceptibility to CNS disease, creating an opportunity to elucidate the antigenic repertoire and the encephalitogenic potential of circulating populations of MBP-reactive T cells.

Here we show that MBP-reactive T cell clones can be derived from the circulation of unimmunized, healthy *C. jacchus*. Furthermore, these T cell clones are consistently able to induce an inflammatory CNS disease by adoptive transfer. Different T

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1. Abbreviations used in this paper: APC, antigen-presenting cells; CNS, central nervous system; CSF, cerebrospinal fluid; EAE, experimental allergic encephalomyelitis; MBP, myelin basic protein; MRI, magnetic resonance imaging; MS, multiple sclerosis; SI, stimulation index.

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cell clones derived from the same animal and reactive against different epitopes of MBP appear to be equally encephalitogenic. The data are the first direct demonstration that T cells reactive against a self-antigen and present in the normal circulating pool are capable of mediating an autoimmune disease.

Methods

Animals. *C. jacchus* marmosets were maintained in primate colonies at the University of California, San Francisco and New England Regional Primate Research Center. The animals used in this study were cared for in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those of the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. A maximum of 2.5 ml of blood every other week was taken from each animal. CSF was obtained by puncture of the cisterna magna. Phlebotomy, cisternal puncture, and intravenous transfer of T cells were done under brief anesthesia (Ketamine, 10 mg i.m.). Magnetic resonance imaging (MRI) was performed under anesthesia with Propofol, 20–50 mg/h in intravenous drip.

Production and characterization of T cell clones. MBP-reactive T cell clones were derived from PBMC of naive (e.g., unimmunized) *C. jacchus* by limiting dilution. Freshly isolated PBMC were cultured at 10^5 cells/well in 96 round-bottom well plates (Corning Inc., Corning, NY) with purified human MBP (50 μ g/ml final concentration) (17) in RPMI 1640 supplemented with 2 mM glutamine (Gibco, Grand Island, NY), 20 mM Hepes buffer and 10% controlled processed serum replacement-2 (CPSR-2) (Sigma Chemical Co., St. Louis, MO), and penicillin/streptomycin/gentamycin at standard concentrations (Gibco). After 3 d the culture medium was supplemented with 10% heat-inactivated human AB serum (Pel-Freez Biologicals, Rogers, AR), 10% human T cell growth factor (Cellular Prods. Inc., Buffalo, NY), 40 U/ml recombinant IL-2 (Hazelton Systems, Inc., Aberdeen, MD) and 4 U/ml IL-4 (Genzyme Corp., Cambridge, MA) and this growth medium was changed every 3 d for 9 d. T cells were restimulated at the end of the cycle by addition of 10^5 syngeneic and/or chimeric freshly isolated PBMC pulsed with MBP (50 μ g/ml) and irradiated (3000 rad) as APC. T cell clones were expanded by multiple cycles of restimulation with MBP/APC followed by culture in growth medium. After 4–6 cycles, MBP-reactive clones were identified and characterized for peptide specificity by [3 H]thymidine incorporation in a 72-h proliferation assay. 5×10^4 T cells were cultured with 10^5 irradiated APC and 25 μ g/ml of MBP, 50 μ g/ml of each MBP-peptide or no antigen (background) in 10% CPSR-2 medium for 72 h. 18 h before termination, [3 H]thymidine (0.5 μ Ci/well) was added. The stimulation index (SI) was measured for each MBP-reactive clone on the day of transfer and was calculated as the ratio of incorporation of [3 H]thymidine (cpm) in the presence of MBP over that of background. Fine mapping of peptide reactivity was studied using a panel of sixteen 20-mer synthetic peptides corresponding to overlapping peptides of human MBP. Human and monkey MBP differ by only 4 of 172 amino acids (18). All peptides recognized by the MBP-reactive T cell clones shared sequence identity with monkey MBP, with the exception of one clone directed against the human 143–162 amino acid sequence that differs by a single amino acid between the species. T cell clones used in control experiments were isolated by the same limiting dilution technique but did not react with MBP or peptides in the proliferation assay.

Adoptive transfer. At the end of the stimulation cycle 10^7 T cells were rested for 48 h in CPSR-2 medium, then restimulated with either: MBP (50 μ g/ml) in the presence of irradiated (3000 rad) syngeneic/chimeric PBMC as APC; or 4 μ g/ml concanavalin A. Control, MBP nonreactive clones were restimulated with 4 μ g/ml concanavalin A. Growth medium was added after 48 h, and 24 h later cells were harvested, washed with RPMI, resuspended in 2 ml of 0.9% saline i.v. and injected into the popliteal vein, followed immediately by intravenous injection of 10^{10} killed *Bordetella pertussis* organisms. 48 h later, a second intravenous injection of *Bordetella pertussis* was administered. In an additional control group, two animals received the two injections of *Bordetella pertussis* but no cells.

Table 1. Effective Cross-presentation of MBP and the Synthetic Peptide 153–172 to the T cell Clone 31.N.2.73 by APC from Chimeric Siblings

Animal source of APC	cpm (SI)		
	Background	MBP (25 μ g/ml)	aa 153–172 (50 μ g/ml)
A*	1,350	15,159 (8.8)	14,761 (9.1)
	1,887	13,498	
B	2,107	13,531 (6.2)	13,112 (5.8)
	2,427	14,943	
C	654	11,835 (17)	15,239 (23)
	679	10,954	

Clone 31.N.2.73 was derived from animal A (*) in chimeric set 3 (see Table II).

Assessment of EAE. The appearance of EAE was monitored in recipient animals by daily clinical evaluation, examination of CSF, MRI, and neuropathologic criteria. The severity of disease was graded by two independent observers blinded as to the identity of the clone transferred: 0, normal; 1, lethargy, anorexia, weight loss; 2, ataxia and either paraparesis/monoparesis, sensory loss or brain stem syndrome including gaze palsy, blindness or dysarthria; 3, paraplegia or hemiplegia; 4, quadriplegia. Postmortem examination was performed on formalin-fixed tissues.

Results

Adoptive transfer of EAE by natural MBP-reactive T cell clones. We first demonstrated that APC from chimeric siblings were equivalent to syngeneic APC in the presentation of MBP or the relevant peptide to the T cell clones derived from one member of the sets (Table I). Therefore, selected clones could be adoptively transferred into the animal of origin or into a chimeric sibling. Table II summarizes the results for five adoptive transfers performed with MBP-reactive clones, and three control adoptive transfers carried out with MBP nonreactive clones (chimeric sets 1–4). In chimeric set 5, animals received intravenous *Bordetella pertussis* but no cells. The SI of the different MBP-reactive clones used for adoptive transfers ranged from 6 to 215. No reactivity to MBP (SI < 1) was detected in the control clones. Clinical signs of EAE developed within 13–27 d after T cell transfer in all five *C. jacchus* that received an MBP-reactive T cell clone (chimeric set 1, animals A and B; chimeric set 2, animal A; chimeric set 3, animals A and B), and in no control animal. Signs included weight loss, sensory loss in the lower limbs, tremor, mono- or paraparesis, or a brain stem syndrome consisting of an altered cry and disordered conjugate gaze. Disease severity did not exceed grade 2 (Table II). In two animals not killed, clinical signs persisted for a period of 4–8 wk before gradually disappearing (animals A and B, chimeric set 3). In one experiment (chimeric set 3, clone 31.N.2.73), concanavalin A appeared as efficient as MBP for stimulation of the encephalitogenic clone prior to transfer. In each animal with EAE, cerebrospinal fluid (CSF) pleocytosis indicative of central nervous system (CNS) inflammatory disease was present at the onset of clinical signs (Table II). Sequential CSF analyses performed in two animals (chimeric set 3) indicated that pleocytosis preceded the onset of neurologic signs (Fig. 1). In the same two animals, MRI studies were performed after the onset of the neurologic signs. Foci of in-

Table II. Adoptive Transfer of EAE by Naturally Occurring MBP-reactive T Cell Clones

Chimeric set	Animal	No. of cells transferred	Clone No. and specificity	SI	Onset	Clinical signs (EAE grade)	CSF	Pathology
					d after transfer		WBC/mm ³	
1	A	10 ⁷	24.N.2.14 aa 153-172 [‡]	52	13	Extraocular motor palsy, dysarthria euthanized day 20 (2)	750	—
	B*	10 ⁷	24.N.2.7 aa 11-30 [‡]	215	14	Paraparesis and sensory loss lower limbs: euthanized day 20 (2)	60	+
	C	10 ⁷	24.N.2.5 Nonreactive [§]	—	(48 d observation)	None: euthanized day 48 (0)	9	—
2	A	10 ⁷	21.N.2.26 aa 143-162 [‡]	21	13	Paraparesis, tremor; euthanized day 22 (2)	360	+
	B	10 ⁷	21.N.2.23 Nonreactive [§]	—	(94 d observation)	None (0)	0	ND
	C	ND	—	—	—	—	—	—
3	A*	10 ⁷	31.N.2.73 aa 153-172 [‡]	8.8	14	Complete sensory loss lower limbs (2)	75	ND
	B	10 ⁷	31.N.2.73 aa 153-172 [‡]	6.3	27	Left lower leg weakness (2)	465	ND
	C	ND	—	—	—	—	—	—
4	A*	10 ⁷	6.N.2.0 Nonreactive [§]	—	(270 d observation)	None (0)	0	ND
	B	10 ⁷	—	—	(72 d observation)	None (0)	0	ND
5	A	i.v. pertussis	—	—	(72 d observation)	None (0)	0	ND
	B	i.v. pertussis	—	—	(48 d observation)	None (0)	ND	ND

* Origin of MBP-reactive T cell clones and control, non-MBP reactive clones used for transfer, e.g., animal B, chimeric set 1; animal C, chimeric set 2 (not shown); animal A, chimeric set 3; animal A, chimeric set 4. Stimulation of T cell clones prior to transfer was performed with either MBP ([‡]) or concanavalin A ([§]). MBP-reactive T cell clones in chimeric sets 1 and 2 were stimulated with MBP. In chimeric set 3, two adoptive transfer were performed with the same clone (31.N.2.73) stimulated with either MBP (animal A) or concanavalin A (animal B). All control, non-MBP-reactive clones were stimulated with concanavalin A. ND, Not done.

creased signal intensity on T2-weighted images were present, suggestive of edema (Fig. 2A). On T1-weighted images, obtained following administration of contrast material, areas of gadolinium enhancement appeared in CNS white matter, indicative of blood brain barrier breakdown (Fig. 2, B and C). Some of the MRI findings in the animals studied correlated with the clinical signs. For example, animal B displayed left lower limb weakness and a lesion within the right side of the midbrain. Animal A had sensory loss in the lower limbs and evidence of a lesion with mass effect in the posterior column of the cervical cord (Table II and Fig. 2, B and C). Control animals were observed for a period ranging between 48 and 270 d. None of the five *C. jacchus* in this group developed clinical signs of

EAE. CSF studies performed at day 14 and 28 after transfer were normal (Table II).

Postmortem examination of the cerebrum and the spinal cord was performed in three animals with EAE and in one control animal. The animals with EAE were killed between day 20 and day 22 after adoptive transfer of T cell clones, corresponding to 6-9 d after the onset of the clinical signs (Table II). At this stage of disease, occasional areas of necrosis and inflammation were present in two of the diseased animals (Table II, Fig. 2D), but foci of demyelination and gliosis were notably absent. No lesion was observed in the control animal studied.

Antigenic repertoire of natural encephalitogenic T cell clones. The frequency of MBP-reactive T cell clones in circulat-

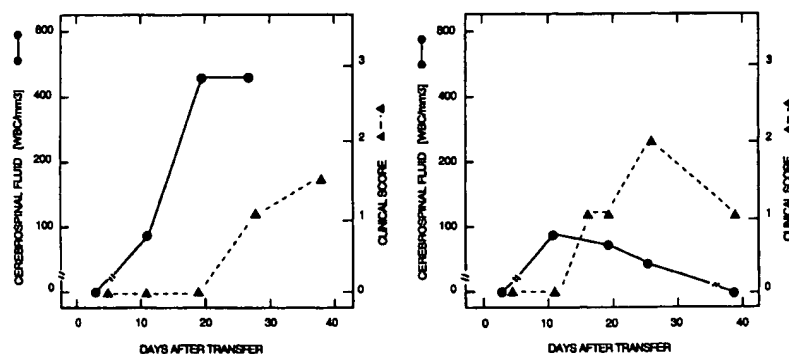


Figure 1. CSF pleocytosis precedes the onset of clinical signs in adoptive EAE. Two animals were studied by sequential cisternal puncture and clinical examination. For each, the CSF cell count (circle) and corresponding clinical score (triangle) is shown in relation to the time of adoptive transfer.

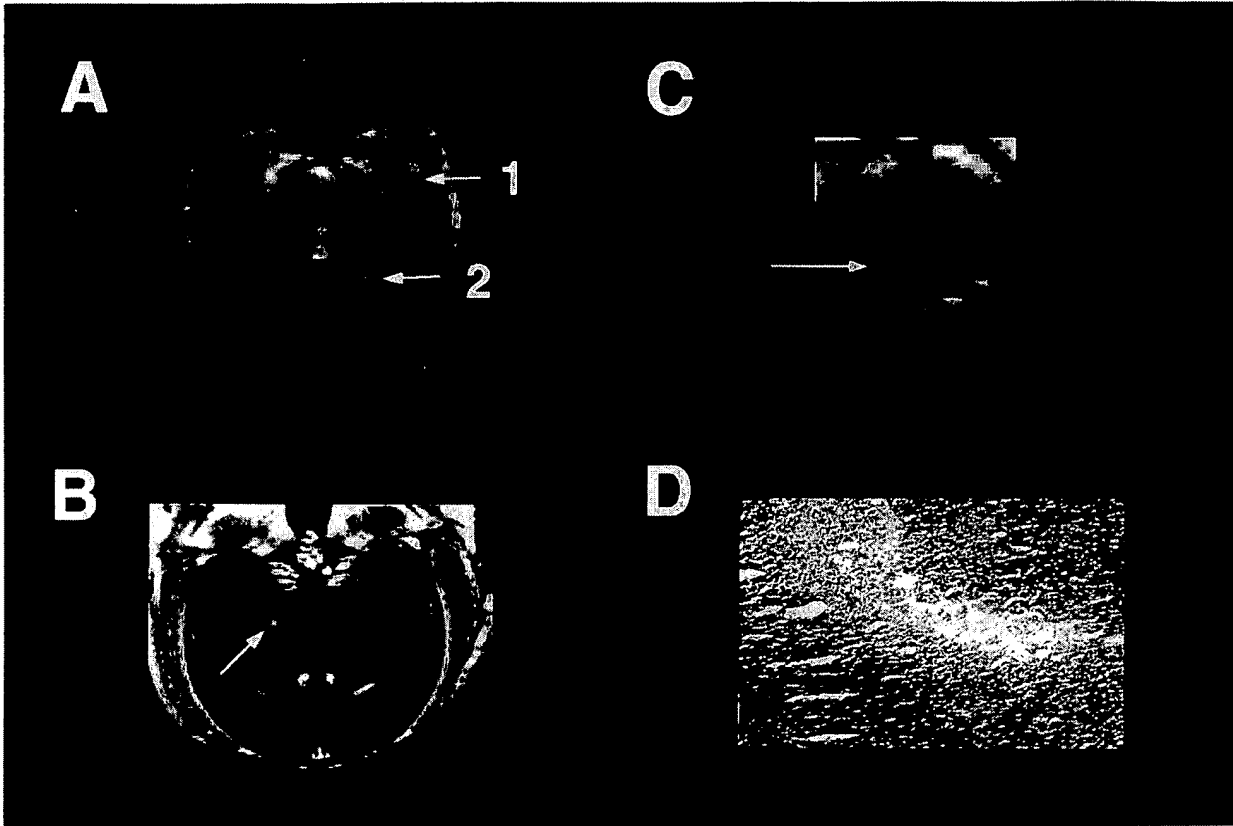


Figure 2. MRI and Pathologic Findings in adoptive EAE. (A) A focus of increased T2 signal intensity appears in the left temporal-parietal white matter (1) and mass effect from a second lesion produces thinning of the left splenium of the corpus callosum (2); (B) A T1 scan following intravenous administration of Gadolinium-DTPA demonstrates an enhancing lesion within the right side of the midbrain (arrow); chimeric set 3, animal B. (C) Cervical cord section demonstrates a T1-Gadolinium enhancing lesion in the right posterior column (arrow) with mass effect on the posterior horn of the gray matter of the cord; chimeric set 3, animal A. (D) Pathological example of a necrotic focus without demyelination in the dorsal mesencephalon. Frozen section. (Hematoxylin/eosin, $\times 300$); chimeric set 2, animal A.

ing PBMC of normal *C. jacchus* ranged between 3 and 7 per 10^{-7} , similar to the frequency estimated to be present in the circulation of healthy humans (8–12). Mapping of the antigenic peptides recognized by individual clones indicated that multiple regions of the MBP molecule were recognized by naturally occurring T cells (Fig. 3). The pattern of reactivity to overlapping peptides identified differences in the fine specificity between clones reactive against similar regions of MBP, further increasing the number of epitopes recognized. For example, the fine specificity of aminoterminal-reactive clones 31N.2.18 and 31N.2.54 could be distinguished by reactivity against aa 1–21 in the former clone only (Fig. 3). Similarly, encephalitogenicity in the blood-derived T cell clones was not restricted by recognition of a single antigenic region of MBP. Four clones, each reactive against one of three different fragments of the molecule (aa 11–30, 143–162, and 153–172), could efficiently transfer disease (Table II). In chimeric set 1, two different clones derived from the same animal, reactive respectively against the amino terminus and carboxyl terminus regions of MBP, were encephalitogenic. These data indicate diversity in the T cell response to MBP that may result in clinical disease.

Discussion

MBP-reactive T cells could readily be cloned from the peripheral blood of normal unimmunized *C. jacchus* primates. They

were similar in several respects to the repertoire of circulating MBP-reactive cells described in human blood (8–12). First, circulating MBP-reactive T cells occur at similar frequencies in the two species. Second, fine specificity mapping of different T cell clones derived from single individuals indicated diversity of recognition to multiple different epitopes of MBP. Thus, both in *C. jacchus* and human primate PBMC, a high frequency of MBP-reactive T cells and diversity in MBP epitope recognition are characteristic.

The bone marrow chimerism in *C. jacchus* made possible the direct demonstration that MBP-reactive T cells were encephalitogenic following expansion and adoptive transfer. Thus, potential disease-inducing populations of MBP-reactive T cells are normally present in primates. In rats, Schluesener and Wekerle were able to isolate a single encephalitogenic MBP-reactive T cell line from lymph node of the EAE susceptible Lewis strain, but a similar line derived from the resistant BN strain was nonencephalitogenic (19). The Lewis line was successfully derived only after multiple negative attempts, and following negative selection of autologous MHC-reactive T cells. This was the first demonstration that EAE-inducing populations are present in the normal immune system. The current data demonstrate that these cells are in relative abundance in the circulation of normal outbred primates and are diverse in terms of their recognition of different epitopes of MBP. In *C. jacchus*, MBP-

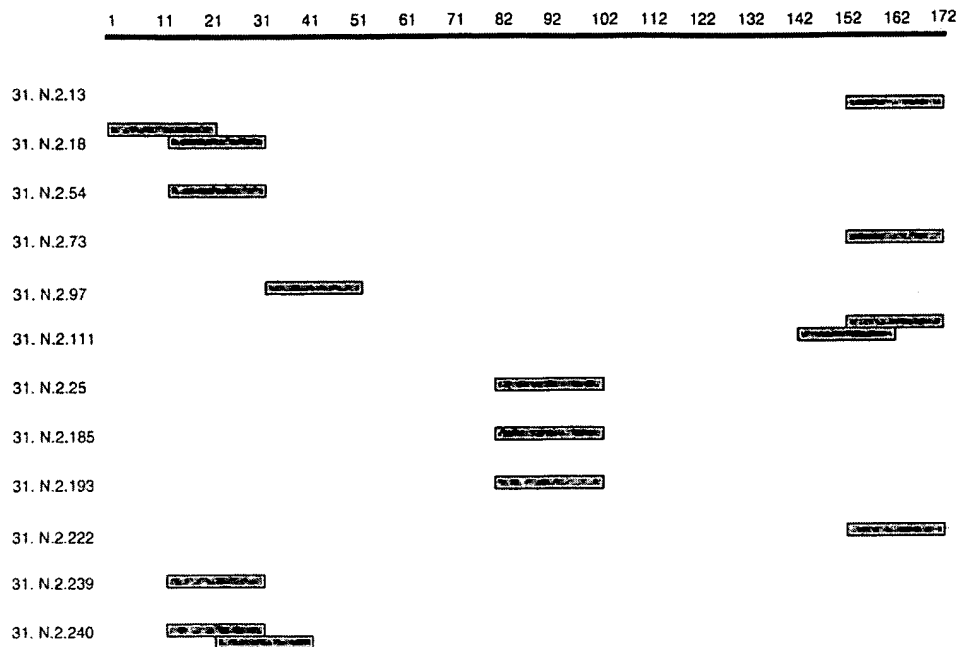


Figure 3. The natural repertoire of circulating MBP-reactive T cells in a healthy *C. jacchus* marmoset. Each clone was mapped for reactivity against synthetic 20-mer peptides corresponding to overlapping regions of human MBP. In this animal (chimeric set 3, animal A) at least seven discrete epitopes of MBP were recognized by different T cell clones.

reactive T cells are neither deleted, nor irreversibly tolerized, in the peripheral pool.

What mechanisms prevent development of spontaneous EAE in *C. jacchus*? MBP-reactive T cells must first penetrate the blood-brain barrier to mediate disease, a process that is influenced by activation (20). Activation by specific antigen, a relevant superantigen (21) or a mitogen (4), could enhance CNS migration of naturally occurring circulating encephalitogenic T cells. The critical role of environmental exposure on the course of EAE was illustrated recently in a transgenic mouse model. A functional T cell receptor derived from an encephalitogenic MBP-reactive T cell was expressed in an MHC compatible host (22). All peripheral immune system CD4 cells expressed the transgenic T cell receptor. Surprisingly, development of EAE in these animals required additional exposure to environmental pathogens or immunization with *Bordetella pertussis*. Transgenic animals unimmunized or housed in a germ-free environment did not spontaneously develop EAE. Thus, a large burden of encephalitogenic T cells may be tolerated under some conditions without pathogenic consequences to the host. This appears also to be the case in *C. jacchus*. It is possible that active suppression might inhibit spontaneous induction of EAE by natural encephalitogenic T cell populations. In Lewis rats tolerized with CNS antigens (23) or recovered from EAE, suppressor T cell lines have been isolated that are capable of preventing EAE after adoptive transfer of encephalitogenic T cell lines (reviewed in 24). In H-2^a mice, recovery from MBP-induced EAE is associated with expansion of a CD4 T cell population that expresses the V β 14 T cell receptor gene segment and responds to a V β 8.2 peptide expressed on most MBP-reactive encephalitogenic T cells in this strain (25).

Mapping data in *C. jacchus* are in agreement with a previous report suggesting that the carboxy terminus of MBP contains

antigenic determinants in primates (26) but also indicates that the natural encephalitogenic repertoire in *C. jacchus* is broad. In most inbred rodents examined, only exceedingly restricted populations of T cells mediate acute EAE. For example, the encephalitogenic response to MBP is largely directed against the 1–9 amino terminus in the H-2^a mouse, against several epitopes within the 89–101 fragment in H-2^b mouse, and against the 68–88 fragment in the Lewis rat (27–29). Similar to *C. jacchus*, diversity in recognition of MBP peptides appears to be characteristic of healthy humans and also patients with multiple sclerosis (MS) (8, 9, 12, 30–33). In outbred primates, both human and nonhuman, heterozygosity at different loci of the MHC may contribute to effective recognition of a greater number of epitopes of MBP than is possible in inbred rodents. Based upon analogy with *C. jacchus*, it is likely that, in humans, T cells that recognize diverse epitopes of MBP are potentially encephalitogenic. This concept has important implications for future attempts to specifically modulate an autoimmune response to MBP in humans.

Prominent and early demyelination is a hallmark of acute EAE induced in *C. jacchus* by active immunization with whole human white matter in adjuvant.² Following adoptive transfer to naive recipients, MBP-reactive T cells induced clinical signs, inflammation in the CSF, and multifocal areas of disruption of the blood-brain barrier on MRI scans. In contrast, however, to the active immunization model, disease severity was mild and demyelination was conspicuously absent in adoptive transfer recipients. This indicates that, in *C. jacchus*, MBP-reactive T cells are capable of inducing CNS inflammation and clinical signs, but are not sufficient for plaque formation. In the active immunization model of EAE, early and intense macrophage infiltration occurs within lesions² and these cells appear to mediate the characteristic demyelination that occurs. This macro-

phage response was not noted following adoptive transfer by MBP-reactive T cells. It is possible that, in synergy with a T cell response to MBP, autoantibodies are required for demyelination to occur in *C. jacchus*. In addition to T cells, a corequirement for antibodies in the pathogenesis of demyelination is present in rabbits (34), guinea pigs (35) and rats (36, 37). It is also likely that, in addition to MBP, other CNS antigens are relevant to EAE in *C. jacchus*, as active immunization with purified human MBP in adjuvant results in a nondemyelinating form of EAE similar to that following adoptive transfer (C. Genain and S. L. Hauser, unpublished observations).

Based upon the finding that primary immune responses to some antigens are not efficiently generated within the CNS, it has long been postulated that the nervous system is immunologically privileged. Relative isolation of the nervous system from the immune system might result in failure to tolerize T cells to CNS antigens, in contrast to antigens expressed in other organs of the body. Naturally occurring autoimmune T cells may exist that mediate encephalomyelitis but not disease in other organ systems. Alternatively, T cells capable of inducing inflammatory disease in many different organs may be generally present in the circulation of primates. In contrast to humans and nonhuman primates, in which naturally occurring MBP-reactive PBMC are present (8, 9, 12, 30–33, 38), we are unaware of any successful identification of MBP-reactive T cells in the circulation of rodents. The *C. jacchus* marmoset is thus remarkable for circulating autoreactive T cells, close phylogenetic similarity to humans, and natural chimerism of bone marrow derived elements, all features unique to this model. These characteristics create an opportunity to study the regulation, traffic, and fate of disease-inducing T cells in normal primate homeostasis.

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References

- Paterson, P. Y. 1960. Transfer of allergic encephalomyelitis in rats by means of lymph node cells. *J. Exp. Med.* 111:119–135.
- Pettinelli C. B., and D. E. McFarlin 1981. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1 + 2 – T lymphocytes. *J. Immunol.* 127:1420–1423.
- Stone, S. H. 1961. Transfer of allergic encephalomyelitis by lymph node cells in inbred guinea pigs. *Science (Wash. DC)*. 134:619–620.
- Panitch, H. S. and D. E. McFarlin. 1977. Experimental allergic encephalomyelitis: enhancement of cell mediated transfer by concanavalin A. *J. Immunol.* 119:1134–1137.
- Johnson, R. T., D. E. Griffin, R. L. Hirsh, J. S. Wolinsky, S. Roedenbeck, I. Lindo de Soriano, and A. Vaisberg. 1984. Measles encephalomyelitis. Clinical and immunologic studies. *N. Engl. J. Med.* 310:137–141.
- Lisak, R. P., P. O. Behan, B. Zweiman, and T. Shetty. 1974. Cell-mediated immunity to myelin basic protein in acute disseminated encephalomyelitis. *Neurology*. 24:560–564.
- Oksenberg, J. R., M. A. Panzara, A. B. Begovich, D. Mitchell, H. A. Erlich, R. S. Murray, R. Shimonkevitz, M. Sherrity, J. Rothbard, C. C. A. Bernard, and L. Steinman. 1993. Selection for T-cell receptor V β -D β -J β gene rearrangements with specificity for a myelin basic protein peptide in brain lesions of multiple sclerosis. *Nature (Lond.)*. 362:68–70.
- Jingwu, Z., R. Madaar, G. A. Hashim, Y. Chin, E. Van den Berg-Loonen, and J. C. M. Raus. 1992. Myelin basic protein-specific T lymphocytes in multiple sclerosis and controls: Precursor frequency, fine specificity and cytotoxicity. *Ann. Neurol.* 32:330–338.
- Joshi, N. K. Usuku, and S. L. Hauser. 1993. The T-cell response to myelin basic protein in familial multiple sclerosis: Diversity of fine specificity restricting elements, and T-cell receptor usage. *Ann. Neurol.* 34:385–393.
- Olsson, T., W. W. Zhi, B. Höjeberg, V. Kostulas, J. Yu-Ping, G. Anderson, H. P. Ekre, and H. Link. 1990. Autoreactive T lymphocytes in multiple sclerosis determined by antigen-induced secretion of interferon- γ . *J. Clin. Invest.* 86:981–985.
- Allegretta, M., J. A. Nicklas, S. Sriram, and R. J. Albertini. 1990. T cells responsive to myelin basic protein in patients with multiple sclerosis. *Science (Wash. DC)*. 247:718–721.
- Ota, K., M. Matsui, E. L. Milford, G. A. Mackin, H. L. Weiner, and D. A. Hafler. 1990. T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature (Lond.)*. 346:183–187.
- Saeiki, Y., T. Mima, S. Sakoda, H. Fujimura, N. Arita, T. Nomura, and T. Kishimoto. 1992. Transfer of multiple sclerosis into severe combined immunodeficiency mice by mononuclear cells from cerebrospinal fluid of the patients. *Proc. Natl. Acad. Sci. USA*. 89:6157–6161.
- Lipton, H. L. 1993. Transfer of multiple sclerosis into severe combined immunodeficiency mice. *Ann. Neurol.* 34:115–116.
- Hao, Q., T. Saida, M. Nishimura, K. Ozawa, and K. Saida. 1994. Failure to transfer multiple sclerosis into severe combined immunodeficiency mice by mononuclear cells from CSF of patients. *Neurology*. 44:163–165.
- Picus, J., W. R. Aldrich, and N. L. Letvin. 1985. A naturally occurring bone marrow chimeric primate. *Transplantation (Baltimore)*. 39:297–303.
- Deibler, G. E., R. E. Martenson, and M. W. Kies. 1972. Large scale preparation of myelin basic protein in central nervous tissue of several mammalian species. *Prep. Biochem.* 2:139–165.
- Tabira, J., and J. I. Kira. 1992. Strain and species differences of encephalitogenic determinants of myelin basic protein and proteolipid apoprotein. In *Myelin: Biology and Chemistry*. R. E. Martenson, editor. CRC Press, Inc., Boca Raton, FL 783–799.
- Schluessener, H. J., and H. Wekerle. 1985. Autoaggressive T lymphocyte lines recognizing the encephalitogenic region of myelin basic protein: in vitro selection from unprimed rat T lymphocyte populations. *J. Immunol.* 135:3128–3133.
- Hickey, W. F., B. L. Hsu, and H. Kimura. 1991. T-lymphocyte entry into the central nervous system. *J. Neurosci. Res.* 28:254–260.
- Brocke, S., A. Gaur, C. Piercy, A. Gautam, K. Gijbels, C. G. Fathman, and L. Steinman. 1993. Induction of relapsing paralysis in experimental autoimmune encephalomyelitis by bacterial superantigen. *Nature (Lond.)*. 365:642–644.
- Goverman, J., A. Woods, L. Larson, L. P. Weiner, L. Hood, and D. M. Zaller. 1993. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. *Cell*. 72:551–560.
- Miller, A., Z. J. Zhang, R. A. Sobel, A. Al-Sabbagh, and H. L. Weiner. 1993. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein. VI. Suppression of adoptively transferred disease and differential effects of oral vs. intravenous tolerization. *J. Neuroimmunol.* 46:73–82.
- Arnon, R., and D. Teitelbaum. 1993. On the existence of suppressor cells. *Int. Arch. Allergy Immunol.* 100:2–7.
- Kumar, V., and E. E. Sercarz. 1993. The involvement of T cell receptor peptide-specific regulatory CD4⁺ T cells in recovery from antigen-induced autoimmune disease. *J. Exp. Med.* 178:909–916.
- Karkhanis, Y. D., D. J. Carlo, S. W. Brostoff, and E. H. Eylar. 1975. Allergic Encephalomyelitis: isolation of an encephalitogenic peptide active in the monkey. *J. Biol. Chem.* 250:1718–1722.
- Zamvil, S. S., D. J. Mitchell, A. C. Moore, K. Kitamura, L. Steinman, and J. B. Rothbard. 1986. T-cell epitope of the autoantigen myelin basic protein that induces encephalomyelitis. *Nature (Lond.)*. 324:258–260.
- Sakai, K., S. S. Zamvil, D. J. Mitchell, M. Lim, J. B. Rothbard, and L. Steinman. 1988. Characterization of a major encephalitogenic T-cell epitope in SJL/J mice with synthetic oligopeptides of myelin basic protein. *J. Neuroimmunol.* 19:821–832.
- Kibler, R. F., R. B. Fritz, F. C.-H. J. Chou, C.-H. J. Chou, N. Y. Peacock, N. M. Brown, and D. E. McFarlin. 1977. Immune response of Lewis rats to peptide C1 (residues 68–88) of guinea pig and rat myelin basic proteins. *J. Exp. Med.* 146:1323–1331.
- Martin, R., D. Jaraquemada, M. Flerlage, J. R. Richert, J. Whitaker, E. O. Long, D. E. McFarlin, and H. F. McFarlin. 1990. Fine specificity and HLA restriction of myelin basic protein-specific cytotoxic T cell lines from multiple sclerosis patients and healthy individuals. *J. Immunol.* 145:540–548.
- Pette, M., K. Fujita, D. Wilkinson, D. M. Altmann, J. Trowsdale, G. Giegerich, A. Hinkkanen, J. T. Epplen, L. Kappos, and H. Wekerle. 1990. Myelin autoreactivity in multiple sclerosis: recognition of myelin basic protein in the context of HLA-DR2 products by T lymphocytes of multiple sclerosis patients and healthy donors. *Proc. Natl. Acad. Sci. USA*. 87:7968–7972.
- Chou, Y. K., M. Vainiene, R. Whitham, D. Bourdette, C. H.-J. Chou, G. Hashim, H. Offner, and A. A. Vandenbark. 1989. Response of human T lympho-

cyte lines to myelin basic protein: Association of dominant epitopes with HLA class II restriction molecules. *J. Neurosci. Res.* 23:207-216.

33. Liblau, R., E. Tournier-Lasserre, J. Maciazek, G. Dumas, O. D. Siffert, G. Hashim, and M. A. Bach. 1991. T cell response to myelin basic protein epitopes in multiple sclerosis patients and healthy subjects. *Eur. J. Immunol.* 21:1391-1395.

34. Brosnan, C. F., G. L. Stoner, B. R. Bloom, and H. M. Wisniewski. 1977. Studies on demyelination by activated lymphocytes in the rabbit eye. II. Antibody-dependent cell-mediated demyelination. *J. Immunol.* 118:2103-2110.

35. Grudke-Igbal, I., C. S. Raine, A. B. Johnson, C. F. Brosnan, and M. B. Bornstein. 1981. Experimental allergic encephalomyelitis: characterization

of serum factors causing demyelination and swelling of myelin. *J. Neurol. Sci.* 50:63-79.

36. Lassmann, H. C. Brunner, M. Bradl, and C. Linington. 1988. Experimental allergic encephalomyelitis: the balance between encephalitogenic T lymphocytes and demyelinating antibodies determines size and structure of demyelinated lesions. *Acta Neuropathol.* 75:566-576.

37. Willenberg, D. O., and S. J. Prowse. 1983. Immunoglobulin-deficient rats fail to develop experimental allergic encephalomyelitis. *J. Neuroimmunol.* 5:99-109.

38. Massacesi, L., N. Joshi, D. Lee-Parritz, A. Rombos, N. L. Letvin, and S. L. Hauser. 1992. Experimental allergic encephalomyelitis in cynomolgus monkeys. Quantitation of T cell responses in peripheral blood. *J. Clin. Invest.* 90:399-404.



Comment in:

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Demyelination in primate autoimmune encephalomyelitis and acute multiple sclerosis lesions: a case for antigen-specific antibody mediation.

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Neuropathological and ultrastructural features of central nervous system demyelination were compared in marmoset experimental autoimmune encephalomyelitis (EAE) induced with myelin/oligodendrocyte glycoprotein (MOG), and in 3 cases of multiple sclerosis (MS) displaying recent lesions. At the edges of EAE and MS lesions, a zone of myelin vacuolation was common, whereas in the lesion proper, myelin sheaths were consistently transformed into vesiculated membranous networks. These networks became dissociated from axons by cell processes from macrophages. Oligodendrocytes were remarkably spared and evidence of myelin repair was present but not prominent. Axonal pathology was more common in the MS material than in marmoset EAE. Immunocytochemistry, using gold-labeled encephalitogenic peptides of MOG and silver enhancement to detect MOG autoantibodies, revealed the presence of MOG-specific autoantibodies over vesiculated myelin networks. Gold-labeled antibody to IgG also gave a positive reaction. Gold-labeled peptide of myelin basic protein did not react with MOG/EAE tissue, but the same conjugate gave positive staining in MS (and in marmoset EAE induced by whole white matter), perhaps indicating broader spectrum immunoreactivity or sensitization to myelin antigens. Thus, vesicular disruption of myelin was a constant feature in these evolving, highly active lesions in primate EAE and MS and appeared causally related to the deposition of antigen-specific autoantibodies.

Identification of autoantibodies associated with myelin damage in multiple sclerosis

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The molecular mechanisms underlying myelin sheath destruction in multiple sclerosis lesions remain unresolved. With immunogold-labeled peptides of myelin antigens and high-resolution microscopy, techniques that can detect antigen-specific antibodies *in situ*, we have identified autoantibodies specific for the central nervous system myelin antigen myelin/oligodendrocyte glycoprotein. These autoantibodies were specifically bound to disintegrating myelin around axons in lesions of acute multiple sclerosis and the marmoset model of allergic encephalomyelitis. These findings represent direct evidence that autoantibodies against a specific myelin protein mediate target membrane damage in central nervous system demyelinating disease.

Multiple sclerosis (MS) is an immune-mediated, relapsing–remitting disorder of the central nervous system (CNS) that results in disabling neurologic deficits in young adults. Pathologically, MS is characterized by perivenular white matter infiltrates comprising macrophages and mononuclear cells (inflammation), and destruction of myelin sheaths that insulate nerve fibers (demyelination). Because of its similarities to the disease model experimental autoimmune encephalomyelitis (EAE), MS is generally thought to be a disorder mediated by T cells. However, the autoantigens that serve as targets for the immune attack have not been identified, and the molecular mechanisms implicated in myelin damage remain uncertain^{1,2}. Although it is obvious that CNS inflammation in EAE is initiated by ‘autoaggressive’ T cells that recognize myelin antigens in the context of class II MHC molecules³, many of the models lack the early demyelinating component of the MS lesion. Indeed, some^{4,5}, but not all⁶ studies have suggested that B-cell activation and antibody responses are necessary for the full development of EAE, and earlier studies on immune-mediated demyelination using myelinated cultures of CNS tissue have indicated that humoral factors are effector mechanisms^{7,8}. The recently developed model of EAE in the common marmoset (*Callithrix jacchus*) has a prominent, MS-like demyelinating component that requires the presence of myelin-specific autoantibodies⁹, and affords an opportunity to investigate how these antibodies interact with their target antigens on myelin. Here, we identify autoantibodies against myelin/oligodendrocyte glycoprotein (MOG) within acute lesions of human MS and *C. jacchus* EAE, where they seem to be directly responsible for the disintegration of the myelin sheaths.

Myelin pathology in *C. jacchus* EAE and in human MS

CNS tissues from six *C. jacchus* marmosets with MOG-induced EAE and from three humans with MS, all with acute lesions (as defined by the presence of cellular infiltrates, demyelination, axonal pathology, hypertrophy of astrocytes and edema), were examined by electron microscopy. In marmoset EAE, large demyelinated plaques up to several millimeters in diameter were disseminated throughout the CNS, always centered on venules

and characterized by perivascular inflammation and a prominent margin along which many myelinated nerve fibers had vacuolated myelin sheaths. This pattern of myelin vacuolation resulted from the enlargement of individual myelin sheaths because of interlamellar splitting and swelling, with the axon displaced to one side surrounded by several layers of intact myelin (Fig. 1a). Between the lesion center and the margin was a broad zone of demyelination containing macrophages laden with myelin debris. The most prominent finding was the presence within the demyelinated zone of large numbers of axons surrounded by aggregates of disrupted myelin rearranged as an expanded network. These axons were displaced laterally as the membranous network gradually became dissociated from the axon and was taken up by adjacent macrophages (Fig. 1b). All three cases of MS had acute lesions in which the pattern of demyelination of fibers was structurally identical to that seen in marmoset EAE, with the demyelinated axon lying within a membranous network of myelin. Elsewhere in the edematous parenchyma, free-floating aggregates of myelin debris were common (Fig. 1c). High-resolution analysis of the myelin networks in both marmoset EAE and human MS demonstrated vesicles surrounded by two to three layers of loosely compacted membranes with a reduced periodicity (5–6 nm) compared with that of intact myelin in normal tissue¹⁰.

MOG-specific autoantibodies and myelin vesiculation in EAE

MOG is a quantitatively minor myelin protein (less than 0.05% of total myelin proteins), with an immunoglobulin (Ig)-like extracellular domain that is expressed in abundance on the outermost layer of myelin sheaths, which may render it accessible to antibody attack¹¹. Although autoantibodies against MOG have been shown to enhance demyelination in several EAE models^{9,12}, the detailed interactions between these antibodies and myelin membranes has not been investigated. To identify the sites of autoantibody binding within demyelinating lesions, we did immunocytochemistry on CNS tissues from MOG-immunized marmosets using gold-labeled anti-monkey or anti-human IgG antibody (both fully cross-reactive with marmoset IgG), followed

by silver enhancement¹³. Positive reactivity for monkey and human IgG and not for the control goat IgG was consistently found over the vesiculated networks of disrupted myelin surrounding axons (Fig. 2a and Table). To identify the target antigens bound by these immunoglobulins, we used immunogold-labeled conjugates of several myelin antigens and control polypeptides. These proteins or short peptides were directly labeled with the gold particles on their primary amino residues and were used to detect antigen-specific autoantibody *in situ*. Mapping studies have established that the demyelinating antibodies present in serum of MOG-immunized marmosets exclusively recognize amino-acid sequences of MOG contained within amino acids (aa) 1–20, 21–40 and 61–80 of this protein (refs. 9,14 and Genain *et al.*, *J. Neuroimmunol.* 90, 34 [Abstr.]). Using the immunogold technique, we found that these three gold-conjugated peptides of MOG were each co-localized over the networks of disintegrating myelin sheaths in a pattern similar to that observed for gold-conjugated anti-IgG (Fig. 2a and c and Table). MOG-reactive droplets were also seen in surrounding macrophages, indicating the presence of internalized myelin debris to which anti-MOG antibody was bound. The immunogold labeling of myelin membranes was specific to the gold-conjugated peptides MOG(aa 1–20), MOG(aa 1–40) and MOG(aa 61–80), as it could be 'quenched' by co-incubating the sections with the corresponding unconjugated peptides (Fig. 2e and Table).

To establish the specificity of the immunogold labeling for the antigenic epitopes of MOG, we did additional immunostaining with the gold conjugate of a peptide of MOG that was not recognized by serum antibodies from the MOG-immunized marmosets included in the study, MOG(aa 101–120). Neither this gold-conjugated, control MOG peptide nor gold conjugates of mouse serum albumin (MSA) aa 560–574 or histone labeled myelin

membranes or macrophages (Fig. 2b, d and f and Table). Similarly, the gold conjugate of myelin basic protein (MBP) aa 82–101, an immunodominant epitope conserved across primate species^{15,16}, did not stain tissues from MOG-immunized marmosets (Fig. 2b and Table). This was not because of the inability of the gold-conjugated peptide MBP(aa 82–101) to label anti-MBP antibodies *in situ*, as this peptide positively stained CNS tissues from whole white matter-immunized marmosets, which are known to de-

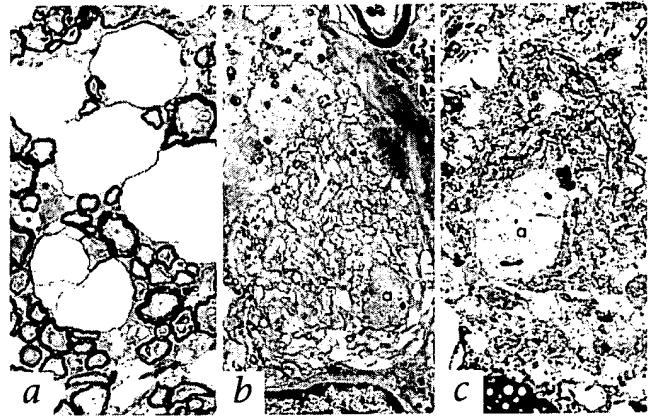
Table Immunogold staining of CNS tissues from *C. jacchus* marmosets with EAE and human subjects with MS or other neurological disorders.

Sample (processing)	Gold conjugate applied	Unconjugated antigen	MOG-immune marmoset serum	Staining	Comments
EAE LESIONS (epoxy) Cases 1 & 2 (MOG-induced)	α-human IgG	-	-	+	vesiculated myelin
	α-monkey IgG	-	-	+	vesiculated myelin
	α-goat IgG	-	-	-	
	MOG(aa 1–20, 21–40, or 61–80)	-	-	+	vesiculated myelin
	MOG(aa 21–40)	MOG(aa 21–40)	-	-	
	MOG(aa 101–120)	-	-	-	
	MBP(aa 82–101)	-	-	-	
	MSA(aa 560–574)	-	-	-	
	histone	-	-	-	
	α-human IgG	-	-	+	vesiculated myelin
Case 3 (WM-induced)	α-goat IgG	-	-	-	
	MOG(aa 21–40)	-	-	+	vesiculated myelin
	MOG(aa 101–120)	-	-	-	
	MBP(aa 82–101)	-	-	++	vesiculated myelin
	MSA(aa 560–574)	-	-	-	
	histone	-	-	-	
MS LESIONS (Epoxy) Case 1 (biopsy)	α-human IgG	-	-	+	vesiculated myelin
	α-goat IgG	-	-	-	
	MOG(aa 1–20, 21–40, or 61–80)	-	-	+	vesiculated myelin
	MBP(aa 82–101)	-	-	+	vesiculated myelin
	MSA(aa 560–574)	-	-	-	
	α-human IgG	-	-	+	vesiculated myelin
	α-goat IgG	-	-	-	
	MOG(aa 21–40)	-	-	+	vesiculated myelin
	MOG(21–40)	MOG(aa 21–40)	-	-	
	MBP(aa 82–101)	-	-	+	vesiculated myelin
Case 2 (autopsy)	α-goat IgG	-	-	-	
	MOG(aa 21–40)	-	-	+	vesiculated myelin
	MOG(21–40)	MOG(aa 21–40)	-	-	
	MBP(aa 82–101)	-	-	+	vesiculated myelin
	histone	-	-	-	
	α-human IgG	-	-	+	vesiculated myelin
Case 3 (autopsy)	α-goat IgG	-	-	-	
	MOG(aa 21–40)	-	-	+	vesiculated myelin
	MOG(aa 21–40)	MOG(aa 21–40)	-	-	
	MBP(aa 82–101)	-	-	+	vesiculated myelin
	histone	-	-	-	
	α-human IgG	-	-	+	vesiculated myelin
CONTROLS (epoxy) ALS (autopsy) Polyglucosan D2 (autopsy)	α-human IgG, MOG(aa 21–40), or MBP(aa 82–101)	-	-	-	
	α-monkey IgG	-	-	+	myelin sheaths
	α-Goat IgG	-	-	-	
	MOG(aa 21–40)	-	-	+	myelin sheaths
EAE NAWM (paraffin)	MOG(aa 21–40)	MOG(aa 21–40)	-	-	
	MOG(aa 101–120)	-	-	-	
	MSA(aa 560–574)	-	-	-	
	histone	-	-	-	
	α-monkey IgG	-	+	++	myelin sheaths
	MOG(aa 21–40)	-	+	++	myelin sheaths
	MOG(aa 21–40)	MOG(aa 21–40)	+	-	
	MOG(aa 101–120)	-	+	-	
	MSA(aa 560–574)	-	+	-	
	histone	-	-	-	

The shaded area shows experiments in which tissue sections were pre-incubated with marmoset immune serum (containing MOG-specific antibodies) before application of the immunogold conjugates. NAWM, normal appearing white matter (spinal cord) with no detectable perivascular inflammatory infiltrate.

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Fig. 1 Electron micrographs of myelin pathology in marmoset EAE and human MS. **a**, Optic nerve from an animal with acute EAE induced by immunization with 50 µg of recombinant rat MOG in adjuvant, killed 3 days after onset of clinical signs. There are large intramyelinic vacuoles at the perimeter of a demyelinated lesion, with axons surrounded by normal-appearing myelin sheaths elsewhere. Original magnification, ×5220. **b**, Lumbar spinal cord from the same animal as in **a**. An Axon (a) is surrounded by disintegrating myelin membranes. Bottom, macrophage; upper left, fully demyelinated axons among vesiculated myelin debris. Original magnification, ×7500. **c**, Biopsy of subcortical white matter from an 18-year-old female patient with an 8 week history of neurologic signs, white matter hypodensity by MRI scan and a diagnosis of acute MS. Myelin around an axon (a) has been transformed into a vesicular network similar to that seen in **b**. Fibrous astroglial processes, naked axons and a reactive, ameboid microglial cell (lower left corner), can also be identified. Original magnification, ×5850. This pattern of myelin breakdown was also a widespread feature of the autopsy cases.



velop anti-MBP antibodies⁹ (Table). These observations demonstrate in this non-human primate model of EAE that antibodies specific to MOG are in direct contact with disintegrating myelin and indicate that formation of the vesiculated membranous networks resulted from lytic attack by these autoantibodies.

MOG peptides detect autoantibodies on normal myelin

The ability of the gold conjugates to stain normal myelin specifically was demonstrated on paraffin sections of apparently uninvolved spinal cord from MOG-immunized marmosets (Fig. 3 and Table). Gold-conjugated anti-monkey IgG, but not gold-conjugated anti-goat IgG, positively stained myelin sheaths and the cytoplasm of oligodendrocytes (Fig. 3a and b), indicating the presence of marmoset IgG bound to the surface of myelinated fibers in these areas of normal-appearing white matter. In addition to staining myelin sheaths, the gold-conjugated anti-monkey IgG sometimes positively stained plasma cells (not shown). As in epoxy-embedded sections containing lesions of EAE, these IgG were specific for immunodominant MOG peptides, as shown by positive staining with the gold conjugates of the MOG antibody epitopes and by 'quenching' with unlabeled antigen (Fig. 3c and d). Furthermore, pre-incubation of tissue with sera from MOG-immunized marmosets accentuated the staining with these gold-conjugated peptides, whereas staining was negative when using the control peptide of MOG(aa 101–120) (Fig. 3e and f). Normal-appearing white matter was not stained with either gold-conjugated histone or MSA(aa 560–574) (Fig. 3g and h). In addition, normal-appearing white matter from control, unimmunized animals was not stained with the gold-conjugates of either anti-monkey IgG or MOG peptides (data not shown). These complementary experiments prove that autoantibodies bound to exposed antigens of myelin could be specifically detected *in situ* using gold conjugates.

MOG-specific autoantibodies and myelin vesiculation in MS

We next investigated, with similar immunogold labeling, the presence of MOG- and MBP-specific autoantibodies in CNS tissue obtained at biopsy or autopsy from three patients with MS (Table). As in marmoset EAE, gold-conjugated anti-human IgG labeled the membranous myelin networks around scattered axons undergoing demyelination, along with droplets of myelin debris displaced throughout the parenchyma (Fig. 4a and Table). Plasma cells also occasionally showed positive staining by anti-IgG. With the immunogold-labeled myelin antigen conjugates, vesiculated myelin networks were intensely stained by gold-con-

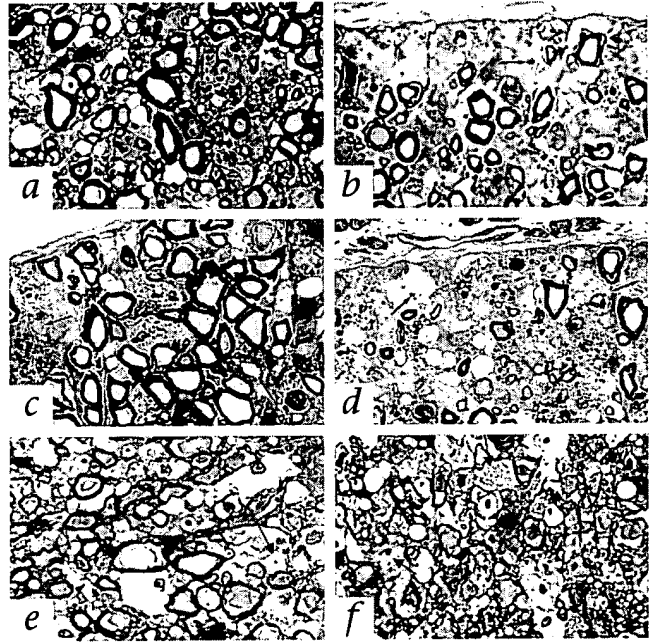
jugated MOG peptides, and to a lesser extent by gold-conjugated MBP, but not by MSA or histone (Fig. 4b–d and f). As with marmoset EAE tissues, positive staining with the gold-conjugated MOG peptides could be eliminated by 'quenching' with unconjugated antigen (Fig. 4e and Table). IgG–myelin complexes labeled with gold-conjugates of MOG and MBP were also present in macrophages but not in astrocytes or oligodendrocytes. No MOG- or MBP-labeled plasma cells were found. Reactivity with gold conjugates was not seen in normal-appearing MS white matter or around perivascular inflammatory cuffs. CNS tissue from cases of amyotrophic lateral sclerosis or polyglucosan D2, two other neurologic disorders associated with white matter damage and macrophage activity, failed to show immunogold reactivity with any of the conjugates (Table). These findings directly identify MOG-specific antibodies in actively demyelinating lesions of human MS, indicating that, as in MOG-induced EAE, these autoantibodies play an integral part in the formation of vesiculated, disrupted myelin sheaths.

Discussion

The structural similarities in the pattern of myelin breakdown between *C. jacchus* EAE and the acute lesions of human MS described here are salient and indicate a common mechanism for myelin destruction. This type of MS lesion represents a form seen in severe acute and primary progressive cases not frequently encountered at autopsy. Within CNS inflammatory lesions, autoantibodies seem to be bound predominantly to the small vesicles that characterize the stage of complete disintegration of the myelin membranes, and to the myelin debris present either in the extracellular space or in phagocytic cells. Similar but less extensive vesiculation of myelin was reported in studies of rodent EAE, in which it was perceived as a transient early phenomenon^{17,18}. However, in the marmoset, in which lesion formation is protracted and ever-expanding, vesiculated myelin was always found. Positive staining of the networks with specific gold-labeled probes provided direct evidence that IgG, and immunoglobulins with MOG-binding capacity, are intimately associated with vesiculated myelin. Here we did not attempt to co-localize immunoglobulins and the MOG protein itself, which would have required additional probes and ultrastructural analysis. However, the finding that both anti-IgG and MOG antigenic peptides (and not control peptides) bind to myelin networks is equally convincing in indicating that autoantibodies are present in these networks and that these antibodies are complexed with MOG.

Although our study shows that anti-MOG antibody may be suf-

Fig. 2 Identification of autoantibodies within marmoset EAE lesions with gold-labeled conjugates. Lumbar spinal cord from the same animal displayed in Figs. 1a and b. Original magnification, $\times 800$. **a**, Immunostaining with gold-conjugated anti-human IgG and silver enhancement. Positive reactivity (brown) is seen on vesiculated myelin around axons (arrows; more apparent around the bottom right axon), indicating the presence of IgG. Most normally myelinated fibers are not stained, although some fibers appear surrounded by a faint ring of brown that is obscured by the counterstain. **b**, Gold-conjugated peptide of MBP. Vesiculated myelin networks are unstained (arrows). **c** and **e**, Gold-conjugated peptide MOG(aa 21–40), whose sequence is conserved across species, in the absence and presence of unconjugated peptide. **c**, Positive reactivity with the labeled antigen indicates the presence of MOG-specific antibody *in situ* on vesiculated myelin around axons (arrows) and on myelin debris within the extracellular space and macrophages. Normal myelin (around most of the fibers) is not stained. **e**, There is no specific staining of myelin membranes in the presence of unconjugated peptide. **d** and **f**, Gold-conjugated peptide of MSA (**d**) and gold-conjugated histone (**f**) also fail to stain vesiculated myelin networks (arrows).



cient to produce myelin vesiculation in MOG-immunized animals, both MOG-specific and MBP-specific immunoglobulins were localized within vesiculated myelin in lesions of MS and EAE in marmosets immunized against whole human white matter (Table). Thus, although anti-MBP antibodies have not been shown experimentally to initiate demyelinating pathology^{9,19–22}, these autoantibodies, and possibly others, could mediate damage to myelin membranes through separate mechanisms such as receptor-mediated phagocytosis by macrophages and/or presentation of myelin autoantigens to specific T cells. Regardless of their respective parts in producing myelin lesions, the origin of the dif-

ferent autoantibodies in MS tissue remains unclear. We did not encounter either MOG-specific or MBP-specific infiltrating B cells here, but such cells have been described in MS brain tissue^{16,23} and in patients with MS, where they seem to be concentrated within the CNS²⁴.

The large-scale vacuolation of myelin at the lesion margin among normally myelinated fibers occurred in the absence of substantial local cellular infiltration or detectable IgG deposition, and has also been reported at the edge of active MS lesions¹. This change in myelin structure could be mediated by soluble factors diffusing from the center of the demyelinating plaque or from activated glial cells at the edge of the lesion^{1,25}. Morphologic changes similar to these large vacuoles have been reported in myelinated CNS cultures exposed to TNF- α ¹ and, to a lesser degree, in CNS cultures exposed to serum from animals with EAE and from subjects with MS (refs 7,8).

It is generally accepted that most humoral immune responses are directed against conformational (or discontinuous) epitopes of proteins rather than against linear amino-acid sequences. In particular, preliminary data indicate that some demyelinating forms of EAE in rodents may be mediated by antibodies directed against discontinuous epitopes of MOG (U. Brehm *et al.*, *J. Neuroimmunol.* 90, 33 [Abstr.]). The precise definition of antibody epitopes of myelin proteins, and especially MOG, in primates is

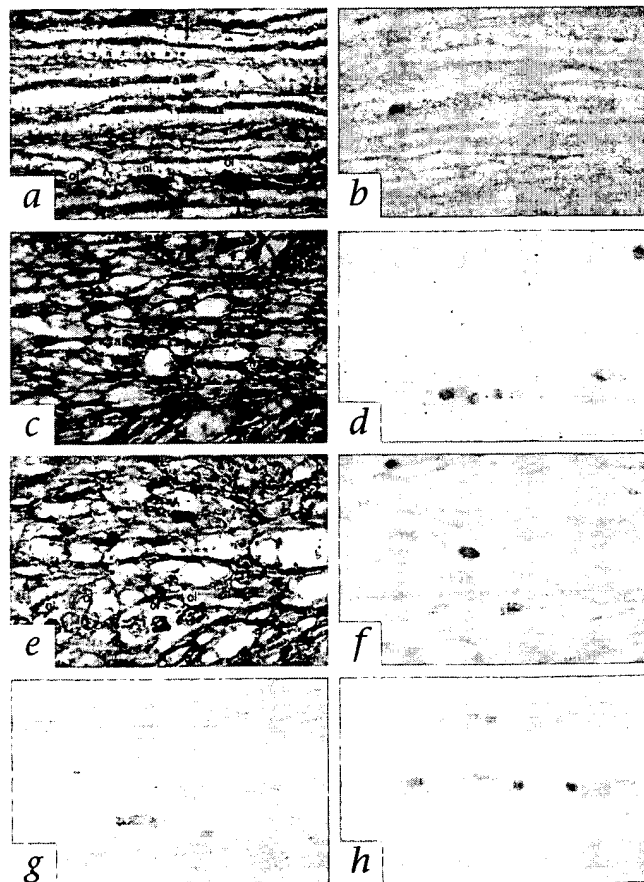
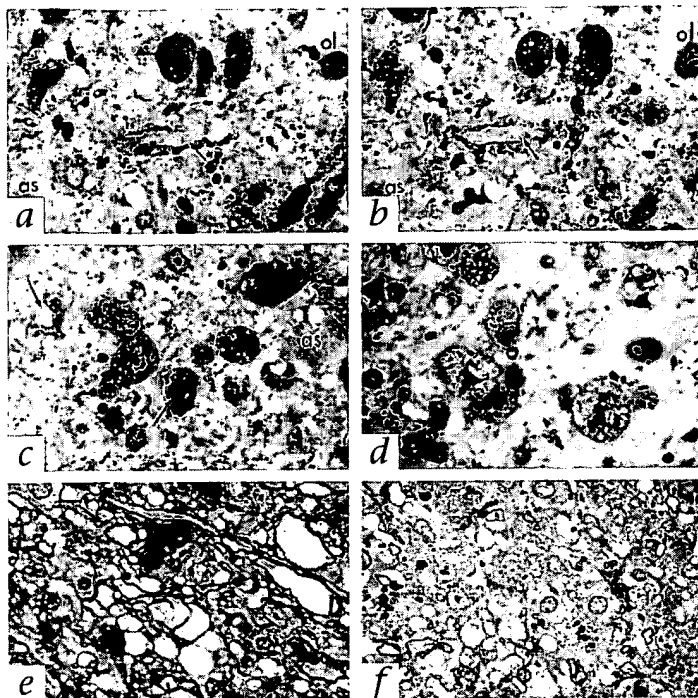


Fig. 3 Staining of normal-appearing white matter with gold-labeled conjugates. Longitudinal sections of paraffin-embedded spinal cord devoid of inflammatory infiltrates, obtained from a MOG-immunized marmoset, were stained with immunogold and counterstained with hematoxylin. Original magnification, $\times 625$. **a** and **b**, Specific staining with gold-conjugated anti-monkey IgG is seen on the surface of intact myelin sheaths (outlined by asterisks) around axons (**a**), and on oligodendrocytes (**ol**). No staining is detected with gold-conjugated anti-goat IgG (**b**). **c** and **d**, Moderate staining of myelin sheaths (asterisks) with the gold conjugate of MOG(aa 21–40) (**c**); staining is totally 'quenched' by pre-incubation with unlabeled MOG(aa 21–40) (**d**). **e–h**, Sections pre-incubated with MOG-immune marmoset serum were stained with the immunogold conjugates. Intense staining is visible with gold-conjugated MOG(aa 21–40) (**e**), but not with gold-conjugates of MOG(aa 101–120) (**f**), histone (**g**) or MSA(aa 560–574) (**h**).

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Fig. 4 Identification of autoantibodies within MS brain tissue from the subject in Fig. 1c. Original magnification, $\times 800$. **a**, Gold-conjugated anti-IgG. IgG is localized along the disintegrated myelin sheath of an axon (arrow) cut in longitudinal section. as, cytoplasm of an hypertrophic astrocyte; ol, tangential section of an oligodendrocyte. Densely stained IgG-coated myelin debris is visible in the parenchyma and, above, in three macrophages (probably ameboid microglia). **b**, Serial section to that in **a**, after immunoreactive labeling with a gold-conjugated peptide of MBP. The same nerve fiber is seen in longitudinal section to demonstrate immunopositivity, albeit less overall than that seen with the MOG conjugate in **c**. Another axon, totally demyelinated, is above the MBP-positive fiber. as, astrocyte; ol, oligodendrocyte. The macrophages (above) and scattered myelin debris also show immunopositivity. **c**, A section adjacent to that in **a**, reacted with gold-conjugated MOG(aa 21–40). Positively-labeled rings of vesiculated myelin around two axons (arrows) cut in cross-section are seen, the center one showing axonal degeneration. Macrophages contain myelin debris and some MOG-positive material. (as), hypertrophic astrocyte. **e**, 'Quenching' is achieved with co-incubation of tissue with unconjugated peptide MOG(aa 21–40). **d** and **f**, Negative staining was obtained with the gold-conjugated peptide of MSA (**d**) and gold-conjugated histone (**f**).



now being investigated. Nonetheless, mapping of antibody responses has been successfully achieved using panels of linear peptides²⁶, and perhaps more importantly, demyelinating forms of EAE can be produced by immunization of animals with short peptidic sequences of MOG, both in rodents^{27,28} and in marmosets (C.G., unpublished observations). Our results show unequivocally that specific autoantibodies bound to myelin can be successfully identified using gold-conjugated peptide antigens (Fig. 3).

Recent work on human MS has emphasized that diverse mechanisms contributing to myelin destruction may result in different forms of MS and heterogeneity of pathology²⁹. Here we focused on antibody-mediated demyelination, and therefore on cases of MS in which the acute lesions were associated with immunoglobulin deposition. Whether this type of lesion represents a particular stage of MS lesions or a separate subtype in severe forms of MS will require additional studies. As for immunoglobulin-associated forms of CNS autoimmune demyelination, we propose the following sequence of events as a mechanism leading to myelin destruction. First, myelin vacuolation is caused by soluble mediators (cytokines, antibodies, free radicals) and/or cellular cytotoxicity¹⁻³. A pattern of intramyelinic edema similar to this has been observed in MS and *in vitro*. Similar patterns of myelin vacuolation have been described in the CNS of rats intoxicated with tri-ethyl tin sulfate³⁰; these changes were reversible. Next, vacuolated myelin is transformed into networks of small vesicles separated by two to three layers of altered myelin with reduced periodicity (5–6 nm). This salient transformation is associated with the deposition of MOG-specific IgG and seems to reflect antibody-mediated damage, possibly because of complement activation, or antibody-dependent cytotoxicity^{1,5,31} mediated by macrophages that are invariably associated with vesicular myelin disruption. The initial vacuolar lesion may render the myelin membranes accessible to attack by autoantibodies. Finally, macrophages are activated, leading to receptor-mediated phagocytosis of the vesiculated myelin debris. This mechanism has been demonstrated in MS and in EAE^{32,33}, with IgG serving as a ligand between the myelin debris and Fc receptors in clathrin-coated pits on the macrophage surface³⁴. This stage of lesion pathogenesis, although antibody-mediated, may be independent

of antibody specificity.

Many of the therapeutic approaches targeting pathogenic T-cell responses in EAE models have not yet translated into successful treatments for human MS, perhaps indicating that other components of the immune system need to be considered. B-cell responses seem to be an essential factor for the severity of clinical disease and pathology in *C. jacchus* EAE (ref. 14). Although antibody-independent mechanisms of autoimmune demyelination exist, our study highlights the potential role of autoantibodies in the widespread destruction of myelin in acute lesions of MS, and emphasizes that in diseases that are initiated by T-cell responses, antibodies against essential antigens of the target organ may be required for the development of irreversible tissue damage.

Methods

Materials. Marmoset CNS tissue was obtained from six animals with MOG-induced demyelinating EAE (refs. 9,14) killed by intracardiac perfusion while sedated by anesthesia, 24–93 days after immunization. Marmosets used for these studies were cared for in full compliance with institutional guidelines. Human CNS tissues were obtained from three subjects with MS, by biopsy or autopsy (8 weeks, 11 years and 17 years after diagnosis). All tissues studied showed acute lesions as defined by the presence of inflammatory infiltrates, ongoing demyelination, macrophages containing recognizable myelin debris, hypertrophic astrocytes, axonal pathology, hyperplasia and edema. Several 'fulminant', intensely demyelinating lesions were available for study from the last two cases.

Electron microscopy. CNS tissue was fixed by perfusion (EAE) or immersion (MS) with 2.5% glutaraldehyde in PO₁ buffer followed by 1% osmic acid, dehydration, and embedding in epoxy resin. Sections were contrasted with lead and uranium salts.

Immunogold labeling. Ultrathin sections of frozen or fixed tissues were used for immunogold labeling. To locate IgG, gold-labeled anti-human IgG and control, gold-labeled anti-goat IgG were used (Nanoprobes, Stony Brook, New York). Gold-conjugated anti-monkey IgG was synthesized by coupling anti-monkey IgG (Sigma) with nanogold particles (Nanoprobes, Stony Brook, New York). To characterize the antigen specificities of IgG, peptides of MOG, MBP and MSA, and purified 21.5-kDa his-

tone (Sigma) were labeled with nanogold particles on their free amino radicals. Peptides were synthesized using the 9-fluorenylmethyloxycarbonyl (Fmoc) N-terminus protecting group for solid phase peptide synthesis and purified (>95%) by HPLC (Research Genetics, Huntsville, Alabama): MOG(aa 1–20), GQFRVIGPRHPRALVGVDEV; MOG(aa 21–40), ELPCRISPGKNATGMEVGVWY; MOG(aa 61–80), QAPEYRGRTLLKDAIGEGK; MOG(aa 101–120), RDHSYQEEAAMELVKVEFPFY; MBP(aa 82–101), QDENPVVHFFKNIVTPRTPP; and MSA(aa 560–574), KPKATAEQLKTVMD. The peptides of MOG were synthesized according to the deduced amino acid sequences of human MOG (ref. 35), which are fully reactive with serum antibodies from MOG-immunized marmosets with the exception of MOG(aa 101–120). All gold conjugates were synthesized using monosulfo-N-hydroxy-succinimide–Nanogold labeling reagent (particle diameter, 1.4 nm) according to the manufacturer's instruction (Nanoprobes, Stony Brook, New York). Gold-conjugated IgG and histone were purified by size exclusion chromatography on PDX G100/50 (Sigma), and gold-conjugated peptides were extensively dialyzed before use to remove unreacted peptide.

Immunoreactivity was detected in standardized assays using epoxy sections 1 µm in thickness. Sections were etched with sodium ethoxide, hydrated, equilibrated in PBS buffer containing 0.05% Triton-X100, and blocked in 10% normal rabbit serum. Sections were then incubated with immunogold conjugates (30 pmol/ml in buffer) for 2 h at room temperature. After washing, staining was detected with Li-Silver enhancement (Nanoprobes, Stony Brook, New York). Sections were counterstained with toluidine blue. 'Quenching' was achieved using unconjugated peptides at concentrations of 4–40 nmol/ml. For paraffin sections, sections of normal-appearing white matter 10 µm in thickness from the spinal cord of MOG-immunized animals were autoclaved for 15 min, deparaffinized and rehydrated in alcohol/H₂O, then PBS. Sections were blocked with 10% normal rabbit serum, and gold conjugates were applied at the same concentrations as above. For those experiments with pre-incubation with MOG-immune marmoset serum, sera were added at a dilution of 1:100. Sections were counterstained with hematoxylin.

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- Brosnan, C.F. & Raine, C.S. Mechanisms of immune injury in multiple sclerosis. *Brain Pathol.* **6**, 243–257 (1996).
- Hohlfeld, R. Biotechnological agents for the immunotherapy of multiple sclerosis. Principles, problems and perspectives. *Brain* **120**, 865–916 (1997).
- Wekerle, H., Linington, C., Lassmann, H. & Meyermann, R. Cellular immune reactivity within the CNS. *Trends Neurosci.* **9**, 271–277 (1986).
- Willenborg, D.O. & Prowse, S.J. Immunoglobulin-deficient rats fail to develop experimental allergic encephalomyelitis. *J. Neuroimmunol.* **5**, 99–109 (1983).
- Piddlesden, S.J., Lassmann, H., Zimprich, F., Morgan, B.P. & Linington, C. The demyelinating potential of antibodies to myelin oligodendrocyte glycoprotein is related to their ability to fix complement. *Am. J. Pathol.* **143**, 555–564 (1993).
- Wolf, S., Dittel, B., Hardardottir, F. & Janeway, C.J. Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. *J. Exp. Med.* **184**, 2271–2278 (1996).
- Raine, C.S. & Bornstein, M.B. Experimental allergic encephalomyelitis: an ultrastructural study of demyelination in vitro. *J. Neuropathol. Exp. Neurol.* **29**, 177–191 (1970).
- Raine, C.S., Hummelgard, A., Swanson, E. & Bornstein, M.B. Multiple sclerosis: serum-induced demyelination in vitro. A light and electron microscope study. *J. Neurol. Sci.* **20**, 127–148 (1973).
- Genain, C.P. et al. Antibody facilitation of multiple sclerosis-like lesions in a non human primate. *J. Clin. Invest.* **96**, 2966–2974 (1995).
- Kirschner, D.A. & Blaurock, A.E. in *Myelin: Biology and Chemistry* (ed. Martenson, R.E.) 3–78 (CRC, Boca Raton, Florida, 1992).
- Brunner, C., H. Lassmann, Waehndt, T.V., Matthieu, J.M. & Linington, C. Differential ultrastructural localization of myelin basic protein, myelin/oligodendroglial glycoprotein, and 2'3' cyclic nucleotide phosphodiesterase in the CNS of adult rats. *J. Neurochem.* **52**, 296–304 (1989).
- Lassmann, H., Brunner, C., Bradl, M. & Linington, C. Experimental allergic encephalomyelitis: the balance between encephalitogenic T lymphocytes and demyelinating antibodies determines size and structure of demyelinated lesions. *Acta Neuropathol. (Berl)* **75**, 566–576 (1988).
- Takisawa, T. & Robinson, J.M. Use of 1.4-nm immunogold particles for immunocytochemistry on ultra-thin-cryo-sections. *J. Histochem. Cytochem.* **43**, 1615–1623 (1994).
- Genain, C.P. et al. Late complications of immune deviation therapy in a non human primate. *Science* **274**, 2054–2057 (1996).
- Tabira, T. & Kira, J.-I. in *Myelin: Biology and Chemistry* (ed. Martenson, R.E.) 783–799 (CRC, Boca Raton, Florida, 1992).
- Warren, K.G., Catz, I. & Steinman, L. Fine specificity of the antibody response to myelin basic protein in the central nervous system in multiple sclerosis: the minimal B cell epitope and a model of its features. *Proc. Natl. Acad. Sci. USA* **92**, 11061–11065 (1995).
- Raine, C.S., Snyder, D.H., Valsamis, M.P. & Stone, S.H. Chronic experimental allergic encephalomyelitis in inbred guinea pigs. An ultrastructural study. *Lab. Invest.* **31**, 369–380 (1974).
- Dal Canto, M.C., Wisniewski, H.M., Johnson, A.B., Brostoff, S.W. & Raine, C.S. Vesicular disruption of myelin in autoimmune demyelination. *J. Neurol. Sci.* **24**, 313–319 (1975).
- Seil, F.J., Falk, G.A., Kies, M.W. & Alvord, E.C. The in vitro demyelinating activity of sera from guinea pigs sensitized with whole CNS and with purified encephalitogen. *Exp. Neurol.* **22**, 545–555 (1968).
- Raine, C.S., Johnson, A.B., Marcus, D.M., Suzuki, A. & Bornstein, M.B. Demyelination in vitro. Absorption studies demonstrate that galactocerebroside is a major target. *J. Neurol. Sci.* **52**, 117–131 (1981).
- Brosnan, C.F., Traugott, U. & Raine, C.S. Analysis of humoral and cellular events and the role of lipid haptens during CNS demyelination. *Acta Neuropathol. (Berl)* Suppl. **9**, 59–70 (1983).
- Linington, C. & Lassmann, H. Antibody responses in chronic relapsing experimental allergic encephalomyelitis: correlation of serum demyelinating activity with antibody titre to the myelin/oligodendrocyte glycoprotein (MOG). *J. Neuroimmunol.* **17**, 61–69 (1987).
- Gerritse, K. et al. The involvement of specific anti myelin basic protein antibody-forming cells in multiple sclerosis immunopathology. *J. Neuroimmunol.* **49**, 153–159 (1994).
- Sun, J. et al. T and B cell responses to myelin oligodendrocyte glycoprotein in multiple sclerosis. *J. Immunol.* **146**, 1490–1495 (1991).
- Benveniste, E.N. Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis. *J. Mol. Med.* **75**, 165–173 (1997).
- Morris, G. in *Epitope mapping protocols* 1–416 (Humana Press, Totowa, 1996).
- Amor, S. et al. Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. *J. Immunol.* **153**, 4349–4356 (1994).
- Ichikawa, M., Johns, T.G., Liu, J. & Bernard, C.C.A. Analysis of the fine B-cell specificity during the chronic/relapsing course of a multiple sclerosis-like disease in Lewis rats injected with the encephalitogenic myelin oligodendrocyte glycoprotein peptide 35–55. *J. Immunol.* **157**, 919–926 (1996).
- Lassmann, H., Raine, C.S., Antel, J. & Prineas, J. Immunopathology of multiple sclerosis: report on an international meeting held at the Institute of Neurology of the University of Vienna. *J. Neuroimmunol.* **86**, 213–217 (1998).
- Aleu, F.P., Katzman, R. & Terry, R.D. Fine structure and electrolyte analysis of cerebral edema induced by alkyl-tin intoxication. *J. Neuropath. Exp. Neurol.* **22**, 403–(1963).
- Compston, A., Scolding, N., Wren, D. & Noble, M. The pathogenesis of demyelinating disease: insights from cell biology. *Trends Neurosci.* **14**, 175–182 (1991).
- Prineas, J.W. & Connell, F. The fine structure of chronically active multiple sclerosis plaques. *Neurology* **28**, 68–75 (1978).
- Epstein, L.G., Prineas, J.W. & Raine, C.S. Attachment of myelin to coated pits on macrophages in experimental allergic encephalomyelitis. *J. Neurol. Sci.* **61**, 341–348 (1983).
- Moore, G.R.W. & Raine, C.S. Immunogold localization and analysis of IgG during immune-mediated demyelination. *Lab. Invest.* **59**, 641–648 (1988).
- Pham-Dinh, D. et al. Characterization and expression of the cDNA coding for the human myelin/oligodendrocyte glycoprotein. *J. Neurochem.* **63**, 2353–2356 (1994).

Articles

TNF neutralization in MS

Results of a randomized, placebo-controlled multicenter study

The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group*

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OBJECTIVE: A double-blind, placebo-controlled phase II study was conducted in 168 patients, most with relapsing-remitting MS, to evaluate whether lenercept would reduce new lesions on MRI.

BACKGROUND: Tumor necrosis factor (TNF) has been implicated in MS pathogenesis, has been identified in active MS lesions, is toxic to oligodendrocytes in vitro, and worsens the severity of experimental allergic encephalomyelitis (EAE) in animals. Lenercept, a recombinant TNF receptor p55 immunoglobulin fusion protein (sTNFR-IgG p55), protects against EAE.

METHODS: Patients received 10, 50, or 100 mg of lenercept or placebo IV every 4 weeks for up to 48 weeks. MRI scans and clinical evaluations were performed at screening, at baseline, and then every 4 weeks (immediately before dosing) through study week 24.

RESULTS: There were no significant differences between groups on any MRI study measure, but the number of lenercept-treated patients experiencing exacerbations was significantly increased compared with patients receiving placebo ($p = 0.007$) and their exacerbations occurred earlier ($p = 0.006$). Neurologic deficits tended to be more severe in the lenercept treatment groups, although this did not affect Expanded Disability Status Scale scores. Anti-lenercept antibodies were present in a substantial number of treated patients; serum lenercept trough concentrations were detectable in only a third. Adverse events that increased in frequency in treated patients included headache, nausea, abdominal pain, and hot flushes.

CONCLUSIONS: Lenercept failed to be beneficial, but insight into the role of TNF in MS exacerbations was gained.

Brief Communications

Onset of multiple sclerosis associated with anti-TNF therapy

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Therapies aimed at inhibiting tumor necrosis factor (TNF), a proinflammatory cytokine implicated in autoimmune disease are effective, especially for rheumatoid arthritis. We report a patient with new onset MS closely associated with the initiation of anti-TNF therapy for juvenile rheumatoid arthritis. It is possible that the inhibition of TNF triggered MS in this individual.

Role of nerve growth factor in experimental autoimmune encephalomyelitis

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The expression of neural regulatory molecules by immune cells that infiltrate the nervous system upon injury may be a mechanism for cross-regulation between the nervous system and the immune system. Several lines of evidence implicate nerve growth factor (NGF) signaling through its receptors (TrkA and p75^{NGFR}) as a potential source of communication between the two systems. We observed changes in NGF mRNA expression and protein secretion by T lymphocytes polarized toward the Th2 phenotype. The presence of NGF did not affect T cell proliferation or cytokine production *in vitro*. Mice treated with NGF by i. p. injection following induction of experimental autoimmune encephalomyelitis, an inflammatory, demyelinating disease of the central nervous system, showed a delayed onset of disease and lower clinical scores during the course of disease. These data suggest a role for NGF signaling in the regulation of the immune response, possibly by enhancing sympathetic innervation of lymphoid tissues.

Key words: Experimental autoimmune encephalomyelitis / Nerve growth factor / Neuroimmunology

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1 Introduction

In recent years, it has become increasingly clear that the physiological systems of organisms are intricately connected. In particular, research has demonstrated a bidirectional link between the immune system and the nervous system. The immune response is subject to modulation by the nervous system, with substantial regulation governed by the sympathetic nervous system. Sympathetic nerve fibers innervate both primary and secondary lymphoid organs and appear to influence the activity of resident immune cells, partially through the release of catecholamine neurotransmitters [1–3]. In return, the function of the nervous system is also subject to modulation by the immune system, particularly in cases of injury or disease of the nervous system. A variety of functional cytokine receptors have been identified on cells of the central nervous system (CNS), as well as on peripheral sympathetic nerves and ganglia [4]. In fact, the cytokine IL-4 was shown to increase nerve growth factor

(NGF) expression and affect cell activity when applied to astrocytes in culture [5]. Our laboratory has recently shown that IL-4 expression occurs in the developing CNS [6]. In addition, immune cells infiltrating the nervous system following damage express classic regulatory molecules of the nervous system, including NGF, its receptors, p75^{NGFR} and tyrosine receptor kinase A (TrkA), and adrenergic receptors [7–10].

NGF is a trophic molecule originally identified and characterized by its functions in the nervous system. NGF is capable of promoting the survival, growth and differentiation of peripheral sympathetic and neural crest-derived sensory neurons and is trophic for certain populations of CNS neurons [11]. In addition, it is essential for the repair of some axons following injury [12]. Two receptors for NGF have been identified: p75^{NGFR}, the low-affinity receptor which bears homology to the TNF superfamily of cytokine receptors, and TrkA, the high-affinity tyrosine kinase receptor which alone or in concert with p75^{NGFR} can result in NGF signaling [13, 14].

NGF signaling has been implicated in the regulation of the immune response. Activated immune cells express NGF and this expression is functionally regulated [7, 8]. Furthermore, TrkA is expressed on and functions to

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Abbreviations: MBP: Myelin basic protein NGF: Nerve growth factor CNS: Central nervous system MS: Multiple sclerosis RT: Reverse transcription

modulate the activity of immune cells in response to NGF [15–17]. In fact, Torcia et al. [17] reported that NGF signaling via TrkA serves as an autocrine survival factor for memory B cells. NGF was identified in the inflamed lungs of mice with allergic bronchial asthma [18]. NGF was reported to augment the Th2 response in this disease model, affecting both cytokine and Ig production. Other researchers reported elevated levels of NGF in patients with several different inflammatory diseases, including SLE, rheumatoid arthritis and multiple sclerosis (MS) [19–21]. Notably, in the cerebrospinal fluid of MS patients, levels of NGF are particularly increased during exacerbations [22].

EAE, a demyelinating disease that parallels MS in several ways, has been a useful tool for studying factors that influence autoimmune demyelination. EAE is a CD4⁺ T cell-mediated attack of the myelin associated with neurons of the CNS. The ensuing disease is characterized by inflammation, demyelination and, in some cases, a relapsing/remitting course, features often associated with MS. As in MS, increased levels of NGF have been observed in inflammatory lesions in the brain of EAE-afflicted rats [23]. The strong correlation between inflammatory episodes of disease and increased levels of NGF production suggests that NGF might affect the pathogenesis of immune-mediated demyelination during the course of EAE, and possibly MS.

To explore the role of NGF during the pathogenesis of EAE, we examined the gene expression and protein secretion of NGF by myelin antigen-specific lymphocytes activated *in vitro* under various conditions. We also examined the effect of NGF on T cell proliferation and cytokine production *in vitro* as well as the effect of administration of exogenous NGF on the clinical outcome of EAE induced by either adoptive transfer of encephalitogenic T cells or by active immunization with myelin antigen in adjuvant. These studies suggest a role for immune-mediated NGF signaling during the pathogenesis of EAE.

2 Results

2.1 NGF mRNA is expressed by lymphocytes and regulated by IL-4

To determine whether infiltrating lymphocytes might be contributing to the increased NGF mRNA production in inflammatory reactions of the CNS, we examined the capacity of lymphocytes to produce NGF mRNA *in vitro*. In our initial experiments, lymphocytes were isolated from the lymph nodes, thymus and spleen of transgenic mice carrying T cells expressing the myelin basic protein (MBP)-specific V α 2.3, V β 8.2 TCR. Eighteen hours after

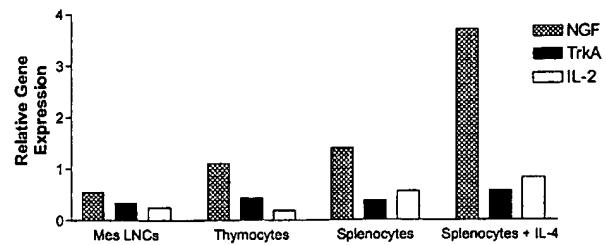


Fig. 1. Gene expression of NGF in lymphocytes from V α 2.3; V β 8.2 TCR-transgenic mice following *in vitro* antigen stimulation. Mesenteric lymph node cells, thymocytes and splenocytes were harvested from naive mice transgenic for T cells expressing the V α 2.3; V β 8.2 TCR and cultured with the MBP Ac1-11 peptide, the antigen recognized by the V α 2.3; V β 8.2 TCR. A subset of splenocytes were cultured with the Ac1-11 peptide and IL-4 (Splenocytes + IL-4). After 18 h in culture, total RNA was analyzed by RT-PCR for expression of IL-2, NGF or TrkA, as indicated. Results are expressed as relative gene expression normalized to the expression of the housekeeping gene HPRT. Baseline values for splenocytes at time 0 were 1.1 for NGF, 0.87 for Trk A and 0.2 for IL-2. Results are representative of three separate experiments.

stimulation with the Ac1-11 peptide of MBP, cells were collected for the measurement of relative gene expression by reverse transcription (RT)-PCR. Lymphocytes from each of the peripheral lymphoid organs produced detectable levels of NGF mRNA (Fig. 1). We chose to examine the effects of IL-4 on the regulation of NGF gene expression in lymphoid tissue since it was known to increase NGF expression in cultured astrocytes. As observed in astrocytes, antigen-stimulated splenocytes activated with MBP Ac-1-11 in culture increased the expression of NGF mRNA significantly in the presence of IL-4 (Fig. 1).

To further examine the regulation of NGF gene expression by IL-4, lymphocytes were harvested from the lymph nodes of V α 2.3, V β 8.2 TCR-transgenic mice. These cells were cultured without antigen, with antigen alone, or with antigen and IL-4. Activated lymphocytes were collected for measurement of relative gene expression by RT-PCR after 6, 18 and 30 h in culture. Eighteen hours after antigen stimulation, NGF mRNA production was only slightly increased in lymphocytes cultured in the absence of antigen or with antigen alone, but was notably increased in the lymphocytes cultured with antigen and IL-4 (Fig. 2).

2.2 NGF protein is expressed by lymphocytes and regulated by IL-4

To determine if the increase in NGF mRNA expression results in an increase in NGF protein secretion, we cultured lymphocytes from transgenic mice with T cells

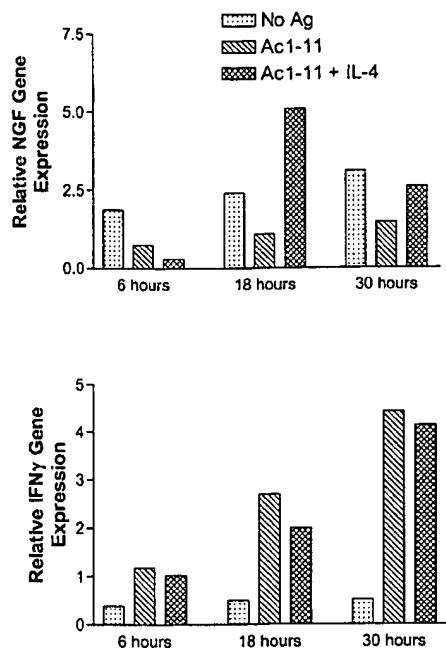


Fig. 2. Increased gene expression of NGF in lymphocytes from Va2.3; Vβ8.2 TCR-transgenic mice following *in vitro* antigen stimulation in the presence of IL-4. Lymph node cells were harvested from naive mice transgenic for T cells expressing the Va2.3; Vβ8.2 TCR and cultured under different conditions. The cells were cultured in media alone (No Ag), with the MBP Ac1-11 peptide (Ac1-11), or with the Ac1-11 peptide and IL-4 (Ac1-11 + IL-4). After 6, 18 and 30 h in culture, total RNA was analyzed by RT-PCR for expression of NGF or IFN-γ, as indicated. Results are expressed as relative gene expression normalized to the expression of the housekeeping gene HPRT. Results are representative of three separate experiments.

expressing MBP-specific Va2.3, Vβ8.2 TCR in the absence of antigen, the presence of antigen, and the presence of antigen and IL-4. When stimulating naive MBP-specific, TCR-transgenic T cells, NGF levels were consistently undetectable prior to 24 h of culture (data not shown). After 72 and 96 h in culture, cell-free supernatants were removed and analyzed for NGF content. As shown in Fig. 3, antigen stimulation alone increased the level of NGF protein in the media nearly fourfold, but this increase was enhanced to nearly tenfold by the presence of both antigen and IL-4.

2.3 NGF protein is expressed by Th2 lymphocytes

To further explore the production of NGF during an immune response, we examined lymphocytes from Vβ8.2 TCR-transgenic mice following either antigen priming or *i. p.* tolerance induction. Following *s. c.* administra-

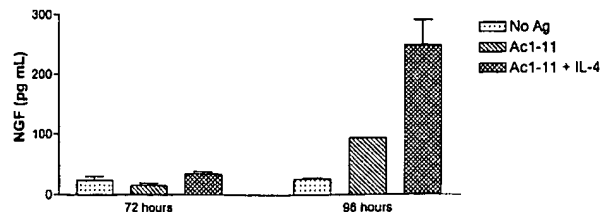


Fig. 3. Induction of NGF secretion by lymphocytes from Va2.3; Vβ8.2 TCR-transgenic mice following *in vitro* antigen stimulation in the presence of IL-4. Lymph node cells were harvested from naive mice transgenic for T cells expressing the Va2.3; Vβ8.2 TCR and cultured under different conditions. The cells were cultured in media alone (No Ag), with the MBP Ac1-11 peptide (Ac1-11), or with the Ac1-11 peptide and IL-4 (Ac1-11 + IL-4). After 72 and 96 h in culture, cell-free supernatants were collected and NGF secretion was measured by ELISA. At 96 h, cells cultured without antigen expressed only 26.3 ± 1.5 pg/ml of NGF, whereas cells stimulated with Ac1-11 expressed 93.9 ± 0.8 pg/ml and Ac1-11 + IL-4 stimulated cells expressed 249.2 ± 34.3 pg/ml of NGF. Results from triplicate wells are shown. Results are representative of three separate experiments.

tion of MBPAc1-11/IFA, the splenocyte production of IFN-γ at 96 h (Fig. 4) indicated these cells had been primed to this antigen [24]. Following *i. p.* administration of antigen, splenocytes produced IL-4 at both 24 and 48 h of stimulation, indicating that *i. p.* antigen administration had resulted in a Th2-like response. The addition of anti-CTLA-4 antibody has previously been shown by our group to potentiate a Th2-like response when given at the time of *i. p.* antigen administration [24]. As illustrated in Fig. 4, both naive cells and antigen-primed cells produce high levels of IFN-γ after 96 h, but relatively little IL-4 or NGF. In contrast, splenocytes from mice receiving *i. p.* antigen produce significant levels of IL-4 and NGF at 24 and 48 h, but relatively little IFN-γ, following *in vitro* stimulation with antigen. Splenocytes from mice receiving *i. p.* antigen continued to produce increased amounts of NGF at 96 h after antigenic stimulation *in vitro*. These data suggest that NGF production occurs in response to Th2-associated stimuli.

2.4 NGF does not affect T cell proliferation and cytokine production *in vitro*

We next examined whether NGF had an effect on T cell proliferation and cytokine secretion. T cells from Va2.3. Vβ8.2 TCR-transgenic mice were cultured with $2 \mu\text{g/ml}$ MBP Ac1-11 and varying concentrations of NGF (0.01–100 ng/ml). As shown in Fig. 5, addition of NGF did not affect T cell proliferation. We next examined whether the presence of NGF *in vitro* affected the cyto-

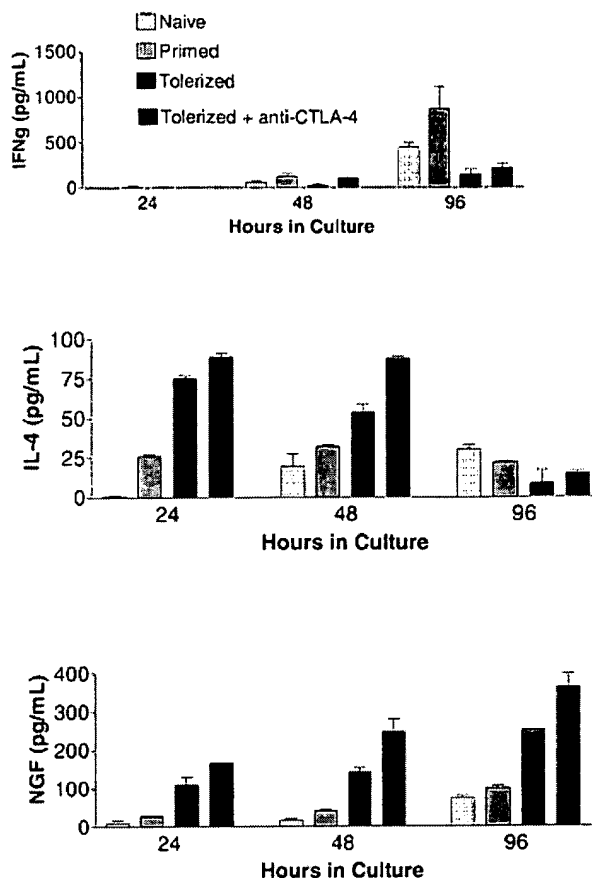


Fig. 4. Regulation of NGF secretion by lymphocytes from Vβ8.2 TCR-transgenic mice following *in vivo* antigen administration. Splenocytes were harvested from mice transgenic for T cells expressing Vβ8.2 TCR 10 days after the mice were unmanipulated (naive), primed via a low dose s.c. injection of MBP Ac1-11 peptide in IFA, tolerized via a high dose i.p. injection of Ac1-11 peptide in IFA, or tolerized via a high dose i.p. injection of Ac1-11 peptide in IFA with concomitant i.p. injection of anti-CTLA-4 antibody. The cells were cultured in media with MBP and cell-free supernatants were collected after 24, 48 and 96 h in culture. NGF, IL-4 and IFN-γ secretion was measured by ELISA. Results from duplicate wells are shown. Results are representative of two separate experiments.

kinase secretion of these MBP Ac1-11-specific T cells. Supernatants were harvested 24, 48 and 72 h after stimulation of MBP-specific T cells in the presence of 10 and 100 ng/ml NGF and examined for the production of IL-4, IL-10 and IFN-γ. Significant levels of IL-4 were not detected. As shown in Fig. 6, no differences in IFN-γ and IL-10 production were noted, suggesting that the levels of NGF produced by the T cells themselves did not dramatically affect the T cell cytokine secretion phenotype *in vitro*. In addition, when T cells were activated in the

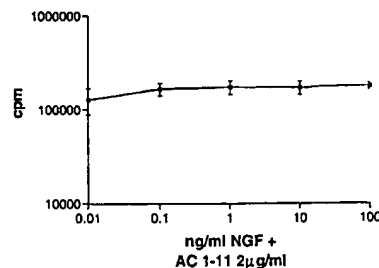


Fig. 5. The presence of NGF does not affect T cell proliferation. Vβ8.2, Vα2.3 TCR-transgenic T cells specific for MBP Ac1-11 were cultured *in vitro* with 2 μg/ml MBP Ac1-11 and various concentrations of NGF (0.01–100 ng/ml). Proliferation was measured by ³H-labeled thymidine incorporation assay. SEM are shown from quadruplicate cultures.

presence of NGF and subsequently transferred into naive recipients, the presence of NGF did not affect the ability of the T cells to transfer EAE (Table 1). These data suggest that NGF alone did not have significant effects on T cell responses *in vitro*.

2.5 NGF treatment ameliorates EAE in mice

Next, we determined whether administration of exogenous NGF would affect the pathogenesis of EAE. We chose to induce EAE in mice transgenic for T cells

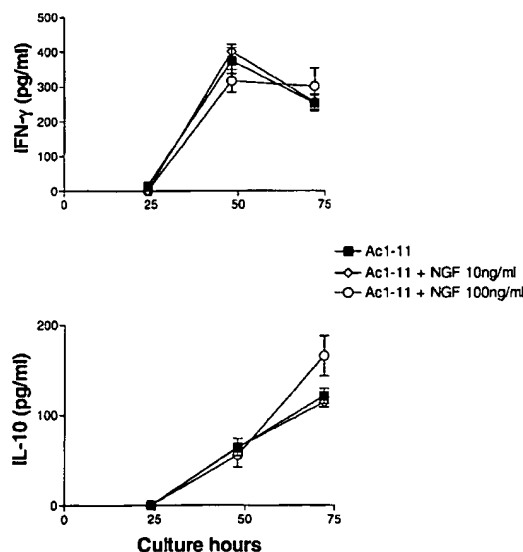


Fig. 6. The presence of NGF does not affect T cell cytokine secretion. Vβ8.2, Vα2.3 TCR-transgenic T cells specific for MBP Ac1-11 were cultured *in vitro* with 2 μg/ml MBP Ac1-11 and 10 and 100 ng/ml NGF. Supernatants were taken at 24, 48 and 72 h and frozen at -20 °C. IL-10 and IFN-γ production was determined by ELISA.

Table 1. Adoptive transfer of EAE with lymphocytes activated in the presence of NGF^{a)}

Activating condition	Incidence	Maximal scores
Ac1-11	5/10	2,2,2,2,1
Ac1-11 + NGF 10 ng/ml	4/10	2,2,2,2
Ac1-11 + NGF 100 ng/ml	6/10	3,2,2,2,2,2

a) Splenocytes from V β 8.2, V α 2.3 TCR-transgenic mice were activated *in vitro* with 2 μ g/ml MBP Ac1-11 for 4 days in the absence or presence of NGF as noted in the table. Activated lymphocytes were transferred into naive, wild-type B10.PL recipients and monitored 45 days for the development of clinical signs of disease. Incidence of EAE and maximal disease scores for individual mice are shown.

expressing the V β 8.2 TCR by direct s.c. immunization with the Ac1-11 peptide of MBP in CFA because no additional manipulations, such as pertussis toxin injection, are necessary for disease induction [24]. Administration of NGF i.p. was initiated on day 1 post immunization and continued on alternate days for 2 weeks. As shown in Fig. 7, animals receiving NGF experienced delayed onset (control 10 ± 2.6 versus NGF 12.5 ± 2 , $p = 0.0065$) and decreased severity of disease ($p < 0.001$ by Mann-Whitney Sum of Ranks). The mean maximal clinical score was 3.8 ± 1.3 in the control mice and 2.8 ± 1.7 in the NGF-treated mice, which was not statistically significant.

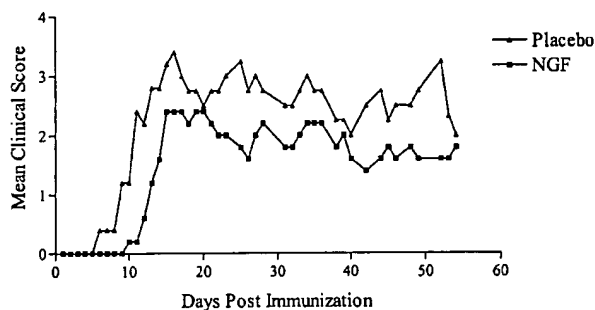


Fig. 7. NGF treatment of mice following induction of EAE by active immunization with the Ac1-11 peptide of MBP. Naive female mice transgenic for T cells expressing the V β 8.2 TCR were immunized with the MBP Ac1-11 peptide in CFA at day 0. NGF (25 μ g) in 0.1 ml saline or 0.1 ml saline alone was injected i.p. on alternating days for 2 weeks beginning on day 1. Mice were monitored daily for clinical disease by a blinded observer. Shown are mean clinical scores ($n = 5$ for each group; $p < 0.001$ by Mann-Whitney Sum of Ranks).

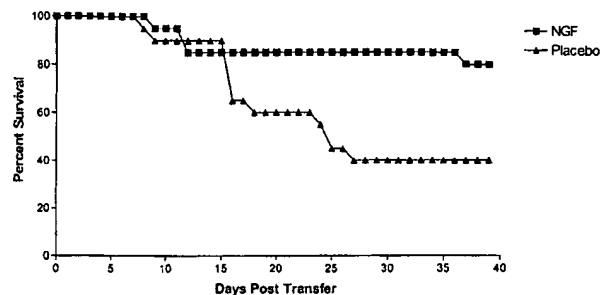


Fig. 8. NGF treatment of mice following induction of EAE by adoptive transfer of encephalitogenic T lymphocytes. Lymphocytes from (PLxSJL) F1 female mice previously immunized with MBP in CFA were activated *in vitro* with MBP, then transferred into naive (PLxSJL) F1 female mice at day 0. NGF (25 μ g) in 0.1 ml saline or 0.1 ml saline alone was injected i.p. on alternating days for 2 weeks beginning on day -1. Mice were monitored daily for clinical disease by a blinded observer. Shown are survival curves for treated (■) and placebo (▲) groups ($n = 20$ for each group; $p < 0.0001$ by t -test).

To further examine whether administration of exogenous NGF would affect the pathogenesis of EAE, we used the adoptive transfer model in (PLxSJL)F1 mice. The administration i.p. of NGF was initiated the day before transfer and continued on alternate days for 2 weeks. Onset of disease was mildly delayed in the NGF-treated mice (control onset on day 9.2 ± 1.4 versus NGF on day 10.6 ± 1.9 , $p = 0.0002$). In this set of experiments, clinical disease was unusually severe (mean maximal score in NGF-treated mice 3.85 ± 1.3 versus 5.1 ± 1.4 in the control mice, $p = 0.0114$). As shown in Fig. 8, of the 20 mice receiving NGF, only three died of severe EAE, while of the 20 mice receiving the saline placebo, 12 died of severe EAE. NGF administration significantly enhanced survival in this model of EAE.

3 Discussion

We have shown that immune cells have the capacity to express NGF and that NGF expression is increased in response to IL-4. It has been shown previously that differentiated CD4⁺ T cells clones (both Th₁ and Th₂ expressed NGF [7, 8, 15]. In our studies, naive T cells stimulated with antigen in the presence of IL-4 expressed significantly higher levels of NGF mRNA and protein than T cells stimulated with antigen alone. In addition, lymphocytes isolated from primed or tolerized transgenic mice produced significant amounts of NGF that correlated with the production of the Th2 cytokine IL-4, but not the Th1 cytokine IFN- γ . These data suggest that CD4⁺ T cells are likely to produce NGF in response

to the presence of IL-4 at the time of antigen challenge *in vivo*.

Interestingly, NGF does not appear to have a dramatic effect on T cell proliferative responses or cytokine secretion *in vitro* (Figs. 5 and 6). However, administration of NGF *in vivo* did appear to have a significant effect on the clinical signs of EAE, suggesting that NGF may be acting through an intermediary, such as the sympathetic nervous system, to modulate an immune response.

Previously, Micera et al. [23] demonstrated that NGF production was increased in the CNS of rats with EAE. NGF may be produced in the CNS during EAE pathogenesis for its neuronal repair function; however, it is also possible that NGF performs an immunomodulatory function as well. Two recent reports have shown that NGF can reduce the antigen presenting capacity of microglial cells *in vitro*, thereby providing a possible mechanism by which NGF production in the CNS during EAE pathogenesis promotes immunomodulation [25, 26]. In an inflamed CNS, NGF-mediated down-regulation of MHC class II expression on microglia would reduce their capacity to present antigen to infiltrating T cells.

In another recent paper by Braun et al. [18], NGF was identified as an important mediator in an allergic airway inflammation model in mice. These studies demonstrated that NGF augmented the production of Th2-associated factors associated with this disease, but not the Th1-associated factors. The increase in gene expression of NGF and TrkA observed in the spinal cord during the pathogenesis of EAE may lead to increased NGF signaling through TrkA as an attempt to augment a Th2 response and quench the ongoing Th1 response. One could hypothesize that NGF contributes to the remission phase of disease by tipping the balance of Th cells in the CNS toward the Th2 phenotype and away from the Th1 phenotype.

If NGF is capable of performing an immunoregulatory function during the course of inflammation in the CNS, it could be used to treat injury and inflammation of the nervous system. Kramer and colleagues previously reported that NGF significantly diminished the pathology associated with experimental autoimmune neuritis, a CD4⁺ T cell-mediated attack of the peripheral nervous system [27]. In addition, the administration of exogenous NGF intracerebrally reduced the clinical signs and neurological symptoms of EAE in a marmoset model [28]. In this study, the authors also suggest that NGF shifts the cytokine response in the CNS from a predominantly Th1 response to a Th2 response. Interestingly, the authors did not note a decrease in the proliferative response of myelin oligodendrocyte glycoprotein-specific lympho-

cytes, which is consistent with our findings (Fig. 5). Furthermore, in the Lewis rat model of EAE, elimination of endogenous NGF using a neutralizing anti-NGF antibody resulted in exacerbation of both the clinical and neuropathological signs of EAE [29].

Here we report that the administration of exogenous NGF ameliorates the clinical severity of EAE. A number of possible mechanisms are available to explain this effect. Because the administration of NGF occurred before the induction of disease and the breakdown of the blood-brain barrier, it is likely that NGF was acting to modulate immune function in the peripheral lymphoid system.

NGF may affect the antigen presentation capability of macrophages or other APC in the lymphoid tissue. Our results showing that NGF did not affect T lymphocyte proliferation would argue against this being a prominent mechanism (Fig. 5). Furthermore, it is also possible that NGF may exert its effects by altering the Th cell phenotype in the periphery rather than in the CNS. NGF administration during EAE induction might cause a shift in polarity away from a Th1 response toward a Th2 response in the peripheral lymphoid organs, thereby ameliorating disease pathogenesis. Similarly, our data suggest that NGF is more likely a product of Th2 cells than Th1 cells.

Similar effects have been observed in studies targeting the role of the sympathetic nervous system on immune function. Alterations in adrenergic signaling in lymphoid tissue disrupts normal immune function [30–35]. Chemical sympathectomy of lymphoid tissue, which effectively removes adrenergic signaling in the tissue, causes exacerbation of EAE in rats [30, 31]. In addition, treatment with β -adrenergic receptor agonists, which mimics sympathetic activity by signaling through adrenergic receptors, actually suppresses both the clinical and histological signs of EAE in rats [32–34]. One possible mechanism by which β -adrenergic signaling regulates the induction of EAE is suggested by a report revealing its differential effects on the activity of polarized Th cells [35]. In this report, exposure of differentiated Th clones to a β -adrenergic receptor agonist prior to antigen stimulation resulted in decreased production of Th1-associated factors, but no change in production of Th2-associated factors. These differences in activity correlated with the expression of the β -adrenergic receptor on the T cell surface, as Th1 cells showed high levels of expression and Th2 cells showed no detectable expression. In the previous studies in which the administration of the β -adrenergic receptor agonist suppressed the induction of EAE, it is possible that the development of pathogenic, antigen-specific Th1 cells was inhibited and only non-pathogenic, antigen-specific Th2 cells were activated. Our data are consistent with a model in which NGF

exerts its regulatory effect not directly on lymphocytes, but rather through the sympathetic nervous system. NGF secretion by activated T cells in the lymphoid tissue would be a stimulus to increase sympathetic regulation.

The results in this study and our prior study using insulin-like growth factor (IGF)-1 to treat chronic, relapsing EAE in mice emphasize the fact that one must consider the effects of these neurotrophic factors on both the nervous system and cells of immune origin [36]. However, unlike the study using IGF-1, which potentiated antigen-specific T lymphocyte proliferation, we found no such effect using NGF (Fig. 5). However, one must consider the pleiotropic effects that NGF can have on various cells of the immune system and CNS if such a strategy is to be used in human diseases such as multiple sclerosis [37]. In summary, our data suggest that neurotrophic factors such as NGF are expressed by cells of the immune system and can participate in the regulation of an immune response such as that which occurs during EAE.

4 Materials and methods

4.1 Mice

Transgenic mice bearing the rearranged V α 2.3;V β 8.2 gene encoding the variable region of the TCR specific for the Ac1-11 peptide of MBP on the B10.PL background were obtained by crossing transgenic mice bearing the individual rearranged genes. The V α 2.3 TCR-transgenic mice and V β 8.2 TCR-transgenic mice were kindly provided by Dr. Joan Goverman (University of Washington, Seattle, WA) [24]. These mice were bred and maintained in our animal colonies at Washington University School of Medicine and University of Texas-Southwestern Medical Center in compliance with the Animal Studies Committee. Female (PLxSJL)F1 mice (4–5 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were 6–10 weeks of age when experiments were initiated.

4.2 Reagents

Whole MBP was prepared from guinea pig spinal cords as previously described and purity was assessed by SDS-PAGE [24]. MBP peptide Ac1-11 was synthesized by the Protein Chemistry Laboratory at Washington University School of Medicine with purity assessed by mass spectrometry. Recombinant mouse IL-4 was purchased from R&D Systems (Minneapolis, MN). Recombinant human NGF was provided by Genentech, Inc. (South San Francisco CA).

4.3 Active immunization

Naive V β 8.2 TCR-transgenic mice were immunized s. c. with MBP Ac1-11 (200 μ g/mouse) in an emulsion with CFA (Difco, Detroit, MI).

4.4 Antigen challenge of mice

At day 0, V β 8.2 TCR-transgenic mice in the primed group were immunized s. c. with MBP Ac1-11 (30 μ g/mouse) in IFA (Difco, Detroit, MI). Mice receiving i. p. tolerization protocols were injected with MBP Ac1-11 (200 μ g)/IFA at day 0. Anti-CTLA-4 mAb (200 μ g) or control hamster IgG (200 μ g) were given on days –1, 0, and +1 relative to i. p. antigen administration.

4.5 Adoptive transfer

Naive (PLxSJL)F1 female mice were immunized s. c. with whole MBP (400 μ g/mouse) in an emulsion with CFA. After 10 days, the mice were killed. Draining lymph nodes were harvested and pressed through a wire mesh screen and combined to obtain a single-cell suspension. The (PLxSJL)F1 or transgenic cells were cultured (4×10^6 cells/ml) in media with 25 μ g/ml whole MBP or 5 μ g/ml MBP peptide Ac1-11, respectively, for 4 days. The activated cells from the (PLxSJL)F1 were suspended in PBS and injected into naive, syngeneic females (3×10^7 cells/mouse). All mice were monitored for clinical signs of disease according to the following scale: 0, no abnormality; 1, a limp tail; 2, moderate hind limb weakness; 3, severe hind limb weakness; 4, complete hind limb paralysis; 5, quadriplegia or premortem state [24].

4.6 Cell culture

Draining lymph nodes, thymus and/or spleens, as noted, from naive, V α 2.3, V β 8.2 TCR-transgenic mice were harvested and single-cell suspensions were obtained by pressing the tissue through a wire mesh screen. The cells were cultured (4×10^6 cells/ml) in complete media for the times specified in the text or figure legends. MBP peptide Ac1-11 (10 μ g/ml) was used as the antigen. When specified, 100 U/ml IL-4 or 10–100 ng/ml NGF was added at the initiation of culture.

4.7 Semi-quantitative RT-PCR

Total RNA was harvested from cells in culture with RNeasy according to the manufacturer's instructions (Tel-Test, Friendswood, TX). RNA was reverse transcribed using the Ready-To-Go T-Primed First-Strand cDNA kit according to manufacturer's instructions (Pharmacia Biotech, Piscataway, NJ). PCR amplification was done in a total volume of 50 μ l containing 5 μ l of the cDNA, 5 μ M of each primer, 200 μ M dNTP (Perkin-Elmer, Foster City, CA) and 1.25 U Taq polymerase (Life Technologies, Gaithersburg, MD) in buffer supplied by the polymerase manufacturer. Primer sequences were: HPRT, 5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3' (sense), 3'-GAT TCA ACT TGC GCT CAT CTT AGG C-5' (antisense); TNF- α , 5'-CTC AAA ATT CGA GTG ACA AGC C-3' (sense), 3'-ACT TGG GCA GAT TGA CCT CAG C-5' (antisense); NGF, 5'-CCA AGG ACG CAG CTT

TCT AT-3' (sense), 3'-CTC CGG TGA GTC CTG TTG AA-5' (antisense); TrkA, 5'-CTC AAC AAG AAG AAT GTG ACG TGC-3' (sense), 3'-GGG TTG AAC TCA AAA GGG TTG TCC-5' (antisense) (Life Technologies, Gaithersburg, MD). Optimum PCR conditions were determined for each set of primers as follows: HPRT, 20 cycles (94° for 60 s, 56° for 60 s, 72° for 1 min, 30 s with a final 10 min at 72°); TNF- α , 25 cycles (94° for 60 s, 56° for 60 s, 72° for 1 min, 30 s with a final 10 min at 72°); NGF, 35 cycles (94° for 60 s, 62° for 40 s, 72° for 1 min, 30 s with a final 10 min at 72°) and TrkA, 35 cycles (94° for 60 s, 62° for 40 s, 72° for 1 min, 30 s with a final 10 min at 72°). The PCR products were run on 1% agarose gels containing ethidium bromide. To provide semi-quantitation of mRNA, the PCR products for all reactions were below detection by ethidium bromide staining to ensure that the saturation point was not reached. The PCR products were transferred from the gel to nylon membranes by capillary transfer for Southern hybridization with a ³²P-labeled internal oligonucleotide probe. Probe sequences were: HPRT, 5'-GTT GTT GGA TAT GCC CTT GAC-3'; TNF- α , 5'-CTC AAA ATT CGA GTG ACA AGC C-3'; NGF, 5'-GTA CAG GCA GAA CCG TAC ACA G-3'; TrkA, 5'-TCA CTC AGT TCT TGG AGT CTG CGC-3'. If necessary, cDNA samples were diluted to give equivalent HPRT signals by phosphorimager analysis of the Southern blots. Using the same cDNA dilutions in the PCR reactions, the resultant signals for TNF- α , NGF and TrkA were each compared to the corresponding HPRT results for determination of relative gene expression [6].

4.8 ELISA

NGF was measured in cell culture supernatants using a commercial ELISA kit and the manufacturer's instructions (Promega, Madison, WI). IFN- γ , IL-4 and IL-10 were measured using ELISA plates (Immuno 2, Dynatech) which were coated with 2 μ g/ml (50 μ l/well) IFN- γ , IL-4 or IL-10 mAb (Pharmingen, San Diego, CA) in 0.1 M carbonate buffer pH 8.2 overnight at 4°C. The plates were blocked with 200 μ l 10% FBS in PBS for 2 h. Tissue culture supernatant (100 μ l) were added at various dilutions titrated to the linear portion of the absorbance/concentration curve in duplicate and incubated overnight at 4°C. After the plates were washed four times with PBS and 0.05% Tween-20, 100 μ l biotinylated anti-cytokine detecting mAb (directed to a different determinant than the first antibody used to coat ELISA plates) at 1 μ g/ml in PBS and 100% FBS were added for 45 min at room temperature. Then 100 μ l of avidin-peroxidase (2.5 μ g/ml) was added and incubated for 30 min. Subsequently, the peroxidase substrate 2,2'-azin-di (3-ethyl-benzthiazolinsulfonate) in 0.1 M citric buffer pH 4.35 in presence of H₂O₂ was added and the absorbance was measured at 405 nm.

4.9 Lymphocyte proliferation

Proliferative responses of splenocytes (2 \times 10⁵ cells/well) from V β 8.2, V α 2.3 TCR-transgenic mice were determined

using 2 μ g/ml MBP Ac1-11 in the presence of various concentrations of NGF as indicated. Cultures were maintained in 96-well flat-bottom microtiter plates for 96 h at 37°C in humidified 5% CO₂/air. The wells were pulsed with 0.5 μ Ci/well [³H] methyl-thymidine for the final 16 h of culture. Cells were harvested on glass fibers and incorporated [³H] methyl-thymidine was measured with a Betaplate counter (Wallac, Gaithersburg, MD). Results were determined as means from quadruplicate cultures and are shown on a log scale with SEM.

4.10 Treatment of EAE with NGF

Mice receiving either s.c. immunization with Ac1-11 peptide of MBP (200 μ g/mouse) in an emulsion with CFA or i.p. antigen-specific cells in sterile PBS at day 0 were given NGF (25 μ g; approx. 1 mg/kg body weight) in PBS or PBS alone beginning at day -1 and continuing on alternate days for 2 weeks. Mice were monitored by a blinded observer for clinical signs of disease according to the scale described previously [24].

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References

- 1 Felten, D. L., Felten, S. Y., Bellinger, D. L., Carlson, S. L., Ackerman, K. D., Madden, K. S., Olschowki, J. A. and Livnat, S., Noradrenergic sympathetic neural interactions with the immune system: structure and function. *Immunol. Rev.* 1987. **100**: 225–260.
- 2 Madden, K. S., Sanders, V. M. and Felten, D. L., Catecholamine influences and sympathetic neural modulation of immune responsiveness. *Annu. Rev. Pharmacol. Toxicol.* 1995. **35**: 417–448.
- 3 Kohm, A. M. and Sanders, V. M., Suppression of antigen-specific Th2 cell-dependent IgM and IgG1 production following norepinephrine depletion in vivo. *J. Immunol.* 1999. **162**: 5299–5308.
- 4 Rothwell, N. J., Luheshi, G. and Toulmond, S., Cytokines and their receptors in the central nervous system: physiology, pharmacology, and pathology. *Pharmacol. Ther.* 1996. **69**: 85–95.
- 5 Brodie, C., Goldreich, N., Haiman, T. and Kazimirsky, G., Functional IL-4 receptors on mouse astrocytes: IL-4 inhibits astrocyte activation and induces NGF secretion. *J. Neuroimmunol.* 1998. **81**: 20–30.
- 6 Lovett-Racke, A. E., Smith, M. E., Arredondo, L. R., Bittner, P. S., Ratts, R. B., Shive, C. L., Forsthuber, T. G. and Racke, M. K., Developmentally regulated gene expression of Th2 cytokines in the brain. *Brain Res.* 2000. **870**: 27–35.
- 7 Ehrhard, P. B., Erb, P., Graumann, U. and Otten, U., Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. *Proc. Natl. Acad. Sci. USA* 1993. **90**: 10984–10988.

- 8 Santambrogio, L., Benedetti, M., Chao, M. V., Muzaffar, R., Kulig, K., Gabellini, N. and Hochwald, G., Nerve growth factor production by lymphocytes. *J. Immunol.* 1994. **153**: 4488–4495.
- 9 Sanders, V. M., Baker, R. A., Ramer-Quinn, D. S., Kaspirowicz, D. J., Fuchs, B. A. and Street, N. E., Differential expression of the beta2-adrenergic receptor by Th1 and Th2 clones: implications for cytokine production and B cell help. *J. Immunol.* 1997. **158**: 4200–4210.
- 10 Ramer-Quinn, D. S., Baker, R. A. and Sanders, V. M., Activated T helper 1 and T helper 2 cells differentially express the beta-2-adrenergic receptor: a mechanism for selective modulation of T helper 1 cell cytokine production. *J. Immunol.* 1997. **159**: 4857–4867.
- 11 Holtzman, D. M., Kilbridge, J., Li, Y., Cunningham, E. T., Jr., Lenn, N. J., Clary, D. O., Reichardt, L. F. and Mobley, W. C., TrkA expression in the CNS: evidence for the existence of several novel NGF-responsive CNS neurons. *J. Neurosci.* 1995. **15**: 1567–1576.
- 12 Varon, S. and Conner, J. M., Nerve growth factor in CNS repair. *J. Neurotrauma* 1994. **11**: 473–486.
- 13 Mallett, S. and Barclay, A. N., A new superfamily of cell surface proteins related to the nerve growth factor receptor. *Immunol. Today* 1991. **12**: 220–223.
- 14 Barbacid, M., The Trk family of neurotrophin receptors. *J. Neurobiol.* 1994. **25**: 1386–1403.
- 15 Ehrhard, P. B., Erb, P., Graumann, U., Schmutz, B. and Otten, U., Expression of functional trk tyrosine kinase receptors after T cell activation. *J. Immunol.* 1994. **152**: 2705–2709.
- 16 Lambiase, A., Bracci-Laudiero, L., Bonini, S., Starace, G., D'Elia, M. M., De Carli, M. and Aloe, L., Human CD4+ T cell clones produce and release nerve growth factor and express high-affinity nerve growth factor receptors. *J. Allergy Clin. Immunol.* 1997. **100**: 408–414.
- 17 Torcia, M., Bracci-Laudiero, L., Lucibello, M., Nencioni, L., Labardi, D., Rubartelli, A., Cozzolino, F., Aloe, L. and Garaci, E., Nerve growth factor is an autocrine survival factor for memory B lymphocytes. *Cell* 1996. **85**: 345–356.
- 18 Braun, A., Appel, E., Baruch, R., Herz, U., Botchkarev, V., Paus, R., Brodie, C. and Renz, H., Role of nerve growth factor in a mouse model of allergic airway inflammation and asthma. *Eur. J. Immunol.* 1998. **28**: 3240–3251.
- 19 Aloe, L., Tuveri, M. A., Carcassi, U. and Levi-Montalcini, R., Nerve growth factor in the synovial fluid of patients with chronic arthritis. *Arthritis Rheum.* 1992. **35**: 351–355.
- 20 Bracci-Laudiero, L., Aloe, L., Levi-Montalcini, R., Galeazzi, M., Schilter, D., Scully, J. L. and Otten, U., Increased levels of NGF in sera of systemic lupus erythematosus patients. *Neuroreport* 1993. **4**: 563–565.
- 21 Scully, J. L. and Otten, U., NGF: not just for neurons. *Cell Biol. Int.* 1995. **19**: 459–469.
- 22 Laudiero, L. B., Aloe, L., Levi-Montalcini, R., Buttinelli, C., Schilter, D., Gillessen, S. and Otten, U., Multiple sclerosis patients express increased levels of beta-nerve growth factor in cerebrospinal fluid. *Neurosci. Lett.* 1992. **147**: 9–12.
- 23 Micera, A., De Simone, R. and Aloe, L., Elevated levels of nerve growth factor in the thalamus and spinal cord of rats affected by experimental allergic encephalomyelitis. *Arch. Ital. Biol.* 1995. **133**: 131–142.
- 24 Ratts, R. B., Arredondo, L. R., Bittner, P., Perrin, P. J., Lovett-Racke, A. E. and Racke, M. K., The role of CTLA-4 in tolerance induction and T cell differentiation in experimental autoimmune encephalomyelitis: i.p. antigen administration. *Int. Immunol.* 1999. **11**: 1881–1888.
- 25 Neumann, H., Misgeld, T., Matsumuro, K. and Wekerle, H., Neurotrophins inhibit major histocompatibility class II inducibility of microglia: involvement of the p75 neurotrophin receptor. *Proc. Natl. Acad. Sci. USA* 1998. **95**: 5779–5784.
- 26 Wei, R. and Jonakait, G. M., Neurotrophins and the anti-inflammatory agents interleukin-4 (IL-4), IL-10, IL-11 and transforming growth factor-beta1 (TGF-beta1) down-regulate T cell costimulatory molecules B7 and CD40 on cultured rat microglia. *J. Neuroimmunol.* 1999. **95**: 8–18.
- 27 Kramer, R., Zhang, Y., Gehrmann, J., Gold, R., Thoenen, H. and Wekerle, H., Gene transfer through the blood-nerve barrier: NGF-engineered neurotrophic T lymphocytes attenuate experimental autoimmune neuritis. *Nat. Med.* 1995. **1**: 1162–1166.
- 28 Villoslada, P., Hauser, S. L., Bartke, I., Unger, J., Heald, N., Rosenberg, D., Cheung, S. W., Mobley, W. C., Fisher, S. and Genain, C. P., Human nerve growth factor protects common marmosets against autoimmune encephalomyelitis by switching the balance of T helper cell type 1 and 2 cytokines within the central nervous system. *J. Exp. Med.* 2000. **191**: 1799–1806.
- 29 Micera, A., Properzi, F., Triaca, V. and Aloe, L., Nerve growth factor antibody exacerbates neuropathological signs of experimental allergic encephalomyelitis in adult Lewis rats. *J. Neuroimmunol.* 2000. **104**: 116–123.
- 30 Chelmicka-Schorr, E., Checinski, M. and Arnason, B. G., Sympathectomy augments the severity of experimental allergic encephalomyelitis in rats. *Ann. N.Y. Acad. Sci.* 1988. **540**: 707–708.
- 31 Chelmicka-Schorr, E., Checinski, M. and Arnason, B. G., Chemical sympathectomy augments the severity of experimental allergic encephalomyelitis. *J. Neuroimmunol.* 1988. **17**: 347–350.
- 32 Chelmicka-Schorr, E., Kwasniewski, M. N., Thomas, B. E. and Arnason, B. G., The beta-adrenergic agonist isoproterenol suppresses experimental allergic encephalomyelitis in Lewis rats. *J. Neuroimmunol.* 1989. **25**: 203–207.
- 33 Muthyala, S., Wiegmann, K., Kim, D. H., Arnason, B. G. and Chelmicka-Schorr, E., Experimental allergic encephalomyelitis, beta-adrenergic receptors, and interferon gamma-secreting cells in beta-adrenergic agonist-treated rats. *Int. J. Immunopharmacol.* 1995. **17**: 895–901.
- 34 Wiegmann, K., Muthyala, S., Kim, D. H., Arnason, B. G. and Chelmicka-Schorr, E., Beta-adrenergic agonists suppress chronic/relapsing experimental allergic encephalomyelitis (CREAE) in Lewis rats. *J. Neuroimmunol.* 1995. **56**: 201–206.
- 35 Sanders, V. M., Baker, R. A., Ramer-Quinn, D. S., Kaspirowicz, D. J., Fuchs, B. A. and Street, N. E., Differential expression of the beta-adrenergic receptor by Th1 and Th2 clones: Implications for cytokine production and B cell help. *J. Immunol.* 1997. **158**: 4200–4210.
- 36 Lovett-Racke, A. E., Bittner, P., Cross, A. H., Carlino, J. A. and Racke, M. K., Regulation of experimental autoimmune encephalomyelitis with insulin-like growth factor (IGF-1) and IGF-1/IGF-binding protein-3 complex (IGF-1/IGFBP3). *J. Clin. Invest.* 1998. **101**: 1797–1804.
- 37 Ransohoff, R. M. and Trebst, C., Surprising pleiotropy of nerve growth factor in the treatment of experimental autoimmune encephalomyelitis. *J. Exp. Med.* 1991. **173**: 1625–1629.

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Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75.

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Members of the nerve growth factor (NGF) family promote the survival of neurons during development. NGF specifically activates the receptor trkA, initiating a signal transduction cascade which ultimately blocks cell death. Here we show that NGF can have the opposite effect, inducing the death of mature oligodendrocytes cultured from postnatal rat cerebral cortex. This effect was highly specific, because NGF had no effect on oligodendrocyte precursors and astrocytes. Other neurotrophins such as brain-derived neurotrophin factor (BDNF) and neurotrophin-3 (NT-3) did not induce cell death. NGF binding to mature oligodendrocytes expressing the p75 neurotrophin receptor, but not trkA, resulted in a sustained increase of intracellular ceramide and c-Jun amino-terminal kinase (JNK) activity, which are thought to participate in a signal transduction pathway leading to cell death. Taken together, these results indicate that NGF has the ability to promote cell death in specific cell types through a ligand-dependent signalling mechanism involving the p75 neurotrophin receptor.

p75 Neurotrophin Receptor Expression on Adult Human Oligodendrocytes: Signaling without Cell Death in Response to NGF

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Oligodendrocytes (OLs) are the primary targets in the autoimmune disease multiple sclerosis (MS). Cell receptors belonging to the tumor necrosis factor receptor (TNF-R) superfamily, such as TNF receptors and fas, are implicated in signaling the injury response of OLs. The p75 neurotrophin receptor (p75^{NTR}), another member of the TNF-R superfamily, has been reported to mediate nerve growth factor (NGF)-induced apoptosis in some neural systems. To address the potential relationship between p75^{NTR} signaling and OL injury, we assayed adult human OLs cultured under several different conditions for p75^{NTR} and tyrosine kinase receptor trkA expression, for NGF-mediated apoptosis, and for NGF-mediated jun kinase (JNK) or nuclear factor (NF) κ B activation. In the current study, we have found expression of p75^{NTR} on cultured adult CNS-derived human OLs but not on other glial cells. TrkA was not detected

on these OLs in any of the culture conditions tested. Treatment of the OLs with varying concentrations of NGF over a range of time periods resulted in no significant increase in numbers of terminal transferase (TdT)-mediated d-uridine triphosphate (UTP)-biotin nick-end labeling positive OLs, whereas significant cell death was observed using TNF- α . Furthermore, unlike TNF- α treatment, NGF treatment did not significantly activate JNK, although both TNF- α and NGF induced nuclear translocation of NF- κ B. These findings contrast with the recent report of NGF-mediated apoptosis in the OLs of neonatal rats matured *in vitro*, which express p75^{NTR} but not trkA (Casaccia-Bonnett et al., 1996), and suggest that, at least in humans, p75^{NTR} signaling may mediate responses other than apoptosis of OLs.

Key words: p75^{NTR}; oligodendrocytes; human; trkA; NGF; apoptosis; multiple sclerosis; JNK; NF- κ B

Oligodendrocytes (OLs) and their myelin membranes are the primary sites of tissue injury in multiple sclerosis (MS), a chronic autoimmune disease of the human adult CNS (for review, see Raine, 1990). The extent of oligodendrocyte loss varies early in the disease, but in the later progressive phase of the disease, OL loss is a constant feature (Brück et al., 1994; Raine, 1994). The precise basis for the selective injury of OLs remains to be clearly defined. Candidate cell-mediated immune effector responses include direct effector–target cell interactions or soluble factors derived from lymphocytes or glial cells.

Target cell receptors belonging to the tumor necrosis factor receptor (TNF-R) superfamily are implicated in signaling the injury response of OLs (Selmaj et al., 1991; Ozawa et al., 1994; D'Souza et al., 1995, 1996). OLs express TNF receptors *in situ* (Dopp et al., 1997), and TNF- α , which is detectable within MS lesions (Selmaj et al., 1991), induces apoptosis of human adult OLs *in vitro* (D'Souza et al., 1995). Human OLs also express fas, both in intact brain and in culture (D'Souza et al., 1996; Bonetti and Raine, 1997), and fas ligand can be detected on glial cells, microglia, and astrocytes (D'Souza et al., 1996, 1998; Dowling et al., 1996; Bonetti and Raine, 1997) and on lymphocytes in MS lesions (D'Souza et al., 1996). Human OLs *in vitro* undergo cell lysis without evidence of nuclear fragmentation in response to

ligation of fas with either fas ligand or with cross-linking antibody (D'Souza et al., 1996).

The precise mechanisms by which TNF and fas receptors mediate death within postmitotic OLs remain unclear, but a conserved intracellular region termed the “death domain” is required for each of these receptors to interact with the downstream signaling elements that are responsible for mediating apoptosis in other cellular systems (for review, see Baker and Reddy, 1996). The p75 neurotrophin receptor (p75^{NTR}), also a member of the TNF-R superfamily, contains a death domain sequence in its cytoplasmic region, and the presence of this motif has fueled speculation that this neurotrophin receptor may play a role in mediating apoptosis *in vivo* (Carter and Lewin, 1997). The neurotrophins [consisting of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4)] are typically considered in terms of their survival-promoting effects that are mediated by neurotrophin-dependent activation of the tyrosine kinase receptors trkA, trkB, and trkC (for review, see Barbacid, 1994). p75^{NTR} was initially found to function as a coreceptor for the trk receptors (Barker and Shooter, 1994; Hantzopoulos et al., 1994; Mahadeo et al., 1994; Verdi et al., 1994), but recent data indicate that p75^{NTR} is also capable of autonomous signaling via neurotrophin-dependent activation of sphingomyelinase activity and nuclear factor (NF) κ B transcriptional complexes (Dobrowsky et al., 1994; Carter et al., 1996). p75^{NTR}-mediated signaling has been implicated in the apoptosis of developing retinal cells (Frade et al., 1996) as well as in the apoptosis of neonatal rat OLs that express p75^{NTR}

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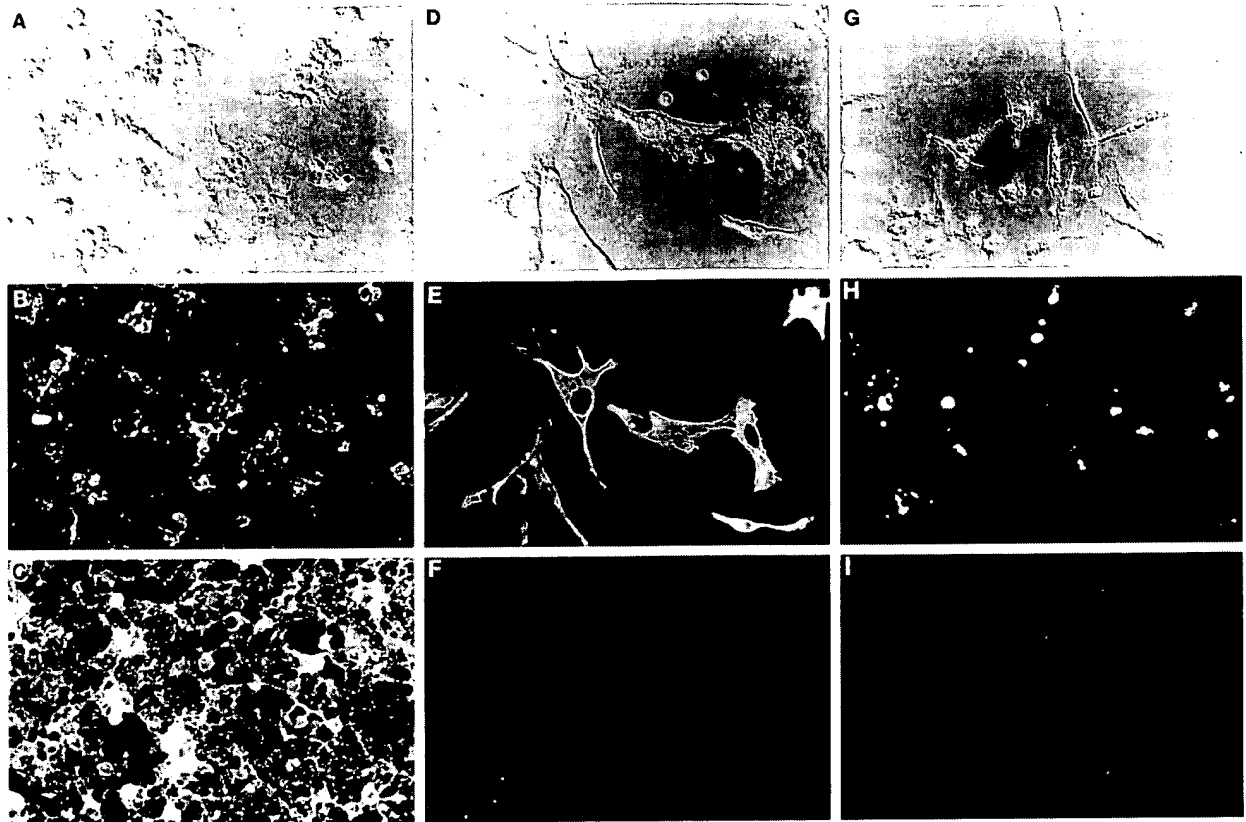


Figure 1. Expression of p75^{NTR} on adult human CNS-derived OLs but not on adult microglia or fetal astrocytes. *A*, Nomarski microphotograph of cultured adult OLs. *B*, OLs stained with O1 [anti-galactocerebroside (Gal C)-O1] antibody. *C*, OLs stained with anti-human p75^{NTR} antibody. *D*, Nomarski microphotograph of cultured human fetal astrocytes. *E*, Fetal astrocytes stained with anti-GFAP antibody. *F*, Fetal astrocytes stained with anti-human p75^{NTR} antibody. *G*, Nomarski microphotograph of cultured adult microglia. *H*, Adult microglia stained with anti-CD68 monoclonal antibody. *I*, Adult microglia stained with anti-human p75^{NTR} antibody. All microphotographs are at a magnification of 500 \times .

but that lack trkA (Casaccia-Bonnet et al., 1996). An effect of p75^{NTR} on OL apoptosis accompanied with increased levels of jun kinase (JNK) activity was observed on neonatal rat OLs cells that were induced to differentiate into mature OLs *in vitro* and maintained in minimal media, but no effect was seen on the immature precursor cells. A separate report indicates that neonatal rodent OLs grown under more typical growth conditions express both trkA and p75^{NTR} and that NGF produces a survival effect rather than apoptosis (Cohen et al., 1996).

The observations that NGF levels are elevated in the CSF of MS patients (Laudiero et al., 1992) and that MS lesions contain both apoptotic OLs (Raine and Scheinberg, 1988; Ozawa et al., 1994; Dowling et al., 1996) and immature OLs with elevated p75^{NTR} expression (Dowling et al., 1997) raise the possibility that p75^{NTR} may play a role in the pathogenesis of MS. To address the potential relationship between p75^{NTR} signaling and OL injury, we cultured adult human OLs under a variety of conditions and assayed them for p75^{NTR} and trkA expression, for NGF-mediated apoptosis, for NGF-mediated JNK activation, and for NGF-mediated NF- κ B nuclear translocation. We have found that cultured adult human OLs express p75^{NTR} and not trkA, and yet, under conditions in which TNF- α results in readily detectable apoptosis and JNK activation, NGF treatment did not result in significant OL apoptosis or JNK activation, although it did induce nuclear translocation of NF- κ B.

MATERIALS AND METHODS

Primary culture preparation. Human brain tissue was obtained from patients undergoing partial temporal lobe resection for intractable epilepsy. A total of 21 such biopsies were used for this study. The glial cell isolation procedure was as described previously (Yong and Antel, 1992). Briefly, brain tissue was subjected to enzymatic dissociation with trypsin (0.25%; GIBCO) and DNase I (25 μ g/ml; Boehringer Mannheim, Indianapolis, IN) for 30 min at 37°C and to mechanical dissociation by passage through a 132 μ m nylon mesh (Industrial Fabrics Corporation, Minneapolis, MN). Mixed glial cells consisting of ~70% OLs, 25% microglia, and 5% astrocytes were obtained by separation on a 30% Percoll (Pharmacia, Dorval, Québec, Canada) gradient (15,000 rpm at 4°C for 30 min). To enrich for OLs, we suspended the mixed-cell population in Eagle's minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS), 2.5 U/ml penicillin, 2.5 μ g/ml streptomycin, 2 mM glutamine, and 0.1% glucose (all from GIBCO) and left the culture overnight in 25 cm² uncoated Falcon tissue culture flasks (VWR Scientific Products) in a humid atmosphere at 37°C and 5% CO₂. The less adherent OLs were removed by gentle shaking. The remaining adherent cells in the Falcon tissue culture flasks, consisting of ~95% microglia and 5% astrocytes, were allowed to develop in MEM with 5% FCS for 2 d. The enriched microglial preparation was then trypsinized (0.25%) and plated onto uncoated 9-mm-in-diameter Aclar coverslips (Seung K, University of British Columbia) or 16-well chamber slides (Nunc, Naperville, IL) for immunocytochemistry.

The OLs were plated onto poly-L-lysine- (10 μ g/ml; Sigma, St. Louis, MO) coated 9-mm-in-diameter Aclar coverslips for immunostaining, onto 16-well tissue culture chamber slides for subsequent immunostaining and cell death assays, or onto 60 mm Petri dishes (VWR Scientific Products) for JNK assays and for reverse transcriptase (RT)-PCR. OLs were initially cultured in MEM with 5% FCS at a density of 5×10^4 cells/coverslip or

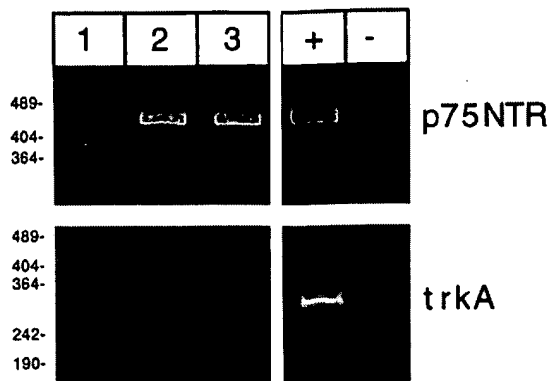


Figure 2. p75^{NTR} mRNA, but not trkA mRNA, is expressed in adult OLs. RT-PCR was performed on RNA extracted from adult OLs grown in DMEM/F12 with PDGF-AA/b-FGF (lane 1), in serum-free MEM (lane 2), or in DMEM/F12 without PDGF-AA/b-FGF (lane 3). Control reactions were performed by substituting OL cDNA with trkA- or p75^{NTR}-expression plasmid (+) or with water (–). The upper panel shows PCR results using p75^{NTR}-specific primers, and the bottom panel shows results obtained using trkA-specific primers. DNA size markers are indicated on the left.

microwell and 1×10^6 cells/ml in the Petri dishes for 7 d. The cells were then washed with serum-free media and cultured under a series of conditions that would permit comparison with previous studies conducted using rodent OLs. Culture conditions used were DMEM/F12 (1:1) supplemented with 50 μ g/ml transferrin, 20 nM progesterone, 50 μ M putrescine, 30 nM selenium, 50 μ g/ml insulin, 0.3% glucose, 15 mM HEPES, 6 mM glutamine, 2.5 U/ml penicillin, and 2.5 μ g/ml streptomycin for 7 d; DMEM/F12 (1:1) with PDGF-AA (20 ng/ml) and b-FGF (20 ng/ml) for 4 d and then transfer to DMEM/F12 without PDGF-AA or b-FGF for 5 d; and serum-free MEM (SF-MEM) for 7 d.

For cell death and JNK assays involving NGF (Collaborative Biomedical Products), TNF- α (Genzyme, Boston, MA), and C2-ceramide (Molecular Probes, Eugene, OR) treatments and for NF- κ B assays using NGF (10 or 100 ng/ml), TNF- α (1000 units/ml), and BDNF and NT-3 (100 ng/ml each; both supplied by Regeneron Pharmaceuticals), cells were washed with serum-free media and incubated in SF-MEM or DMEM/F12 (1:1) containing 0.1% bovine serum albumin (DMEB) and supplemented with the above effector molecules as indicated in the figure legends. NGF-dependent neurite outgrowth from pheochromocytoma (PC12) cells was performed using 10 and 100 ng/ml NGF to ensure that the NGF used in all experiments was bioactive (data not shown).

Because pure cultures of adult astrocytes could not be obtained, fetal human astrocytes were used as an alternative for immunocytochemical studies of p75^{NTR} expression. These were prepared from human fetal CNS tissue obtained at 12–16 weeks of gestation following Medical Research of Canada guidelines. The tissues were mechanically dissociated with scalpel blades and then treated with trypsin (0.25%) and DNase (50 μ g/ml) at 37°C for 45 min. Dissociated tissue was passed through a 130 μ m mesh, washed twice in PBS, and the cells, consisting of astroglia, neurons, and sparse microglia, were plated directly onto poly-L-lysine-coated 75 cm² Falcon tissue culture flasks in MEM with 5% FCS. After confluence, cultures were split using 0.25% trypsin. Populations of proliferating fetal astrocytes were obtained after three to four passages and plated onto uncoated 9 mm Aclar coverslips or 16-well chamber slides.

Immunocytochemistry. OLs were identified by staining live cells with undiluted supernatant of the O1 hybridoma (gift from Dr. W. Yong, Calgary, Canada) for 1 hr at room temperature (RT) followed by three washes in PBS and biotinylated-goat anti-mouse IgG (1:100; Caltag, South San Francisco, CA) for 1 hr. After three rinses in PBS, cells were incubated with Cy3-conjugated Streptavidin (1:1000; Jackson ImmunoResearch, West Grove, PA) for 15 min at RT. After rinsing again with PBS, coverslips were mounted with gelvatol. The cells were also identified as mature OLs by immunostaining methanol–acetone (1:1)-fixed cells with anti-myelin basic protein (MBP) monoclonal antibody (mAb) (Boehringer Mannheim) (data not shown). Microglia were identified using anti-CD68 mAb (1:150; 1 hr at RT; Dako, Carpinteria, CA), and permeabilized fetal astrocytes were identified using rabbit anti-glial

fibrillary acidic protein (GFAP; Dako). For p75^{NTR} staining, anti-human p75^{NTR} (Boehringer Mannheim) was used at a dilution of 1:4000 for 1 hr at RT followed by biotinylated-goat anti-mouse IgG and Cy³-conjugated Streptavidin. To detect nuclear translocation of NF- κ B, we fixed OLs with 4% paraformaldehyde for 20 min at RT and followed with acetone–methanol (1:1) at –20°C for 10 min. They were then stained with a rabbit polyclonal antibody specific for the p65 subunit of NF- κ B (1:100; Santa Cruz Biotechnology, Tebu, France) for 1 hr at RT, followed by biotinylated-goat anti-rabbit IgG (1:100; Caltag) for 30 min and Cy³-conjugated Streptavidin (1:1000) for 15 min at RT. For nuclear DNA staining, Hoechst 33258 (1:1000; Molecular Probes) was used on the same cells. After rinsing with PBS, coverslips were mounted with gelvatol. All slides were examined under a Reichert 2 Polyvar Leica epifluorescent microscope.

RT-PCR analysis. Total RNA was isolated from cultured OLs grown in the different culture conditions described above, using the one-step guanidinium isothiocyanate procedure (Chomczynski and Sacchi, 1987). Total RNA (2.5 μ g) was used for first-strand cDNA synthesis using SuperScript II RNase H-reverse transcriptase (GIBCO BRL) in 20 μ l reactions containing 20 mM Tris-HCl, pH 8.4, 0.5 mM deoxynucleotides, 50 mM KCl, 2.5 mM MgCl₂, 10 mM dithiothreitol, and 50 ng of random hexamers (GIBCO BRL). Five microliters of the resulting cDNA reaction mixture were then used for nested PCR analysis. The initial PCR was performed for 35 cycles at 94°C \times 40 sec, 52°C \times 60 sec, and 72°C \times 90 sec, and the second PCR was performed using 2.5 μ l of the first reaction mixture for 25 cycles at 94°C \times 40 sec, 52°C \times 60 sec, and 72°C \times 90 sec. Products were analyzed on Tris-borate EDTA (TBE) acrylamide gels stained with ethidium bromide. PCR primers used for the first p75^{NTR} PCR were p75^{NTR}-1 (CTC ACA CCG GGG ATG TG) and p75^{NTR}-2 (GTG GGC CTT GTG GCC TAC), and the nested primers for the second were p75^{NTR}-3 (TGT GGC CTA CAT AGC CTT C) and p75^{NTR}-4 (ATG TGG CAG TGG ACT CAC T). For trkA PCR, the initial PCR used primers trkA-1 (ATG AGA CCA GCT TCA TC) and trkA-2 (GCT GTG CTG GCG CCA GA), and the nested primers for the second were trkA-3 (AA CAA CCG CAA CTA CAC) and trkA-4 (CTT GTT TCT CCG TCC AC). The expected final product sizes from cDNA are 476 base pairs for p75^{NTR} and 279 base pairs for trkA; each of the primer sets span exon and intron boundaries, and genomic fragments are larger than the cDNA sizes indicated above.

Cell death assay: TUNEL staining. After exposure to putative injury mediators, cell cultures in 16-well chamber slides were washed with PBS, fixed with acetone–methanol (1:1) for 15 min at –20°C, and then rehydrated with PBS for 30 min at RT. Cells were then incubated with 50 μ l of nick end-labeling solution containing TdT (9.5 units/ml; Promega, Madison, WI) and biotinylated dUTP (10 nmol per ml; Boehringer Mannheim) in terminal transferase (TdT) 5 \times buffer [500 mM cacodylate buffer, pH 6.8, 5 mM CoCl₂, and 0.5 mM DTT (Promega)] for 1 hr at 37°C. The reaction was terminated by adding 10 mM Tris-HCl, pH 6.8, for 5 min at RT. After blocking for 5 min with HHG (1% HEPES, 2% horse serum, and 10% goat serum in HBSS; all from GIBCO), the cells were incubated with Streptavidin-FITC (1:50; 30 min at 37°C; Boehringer Mannheim). To identify cell nuclei, we stained cells simultaneously with propidium iodide (PI; 10 μ g/ml; 10 min incubation). Slides were washed in PBS and mounted for counting. Each experiment was conducted on cells derived from at least three different brain specimens. For each experiment, all treatments were performed in replicate (three or four) wells, and 200–400 cell nuclei per well were counted using a Reichert Polyvar 2 Leica epifluorescent microscope.

JNK assay. Cells grown in each of the culture conditions described above were washed in DMEM containing 0.1% BSA and then treated with putative injury mediators (NGF at 100 ng/ml, TNF- α at 10 ng/ml, and C2-ceramide at 25 μ M) for the times indicated in the figure legends. **In vitro** kinase assays with whole-cell lysates were performed as described in Westwick and Brenner (1995) using Sepharose-conjugated glutathione transferase (GST-jun (1–223) as substrate. Briefly, 15 μ g of cell lysate was incubated for 2 hr at 4°C with the Sepharose-coupled substrate. After extensive washes, *in vitro* kinase assays were performed at 30°C for 20 min. Sepharose-associated phosphorylated substrate was resuspended in sample dissociation buffer and run on 12% SDS-PAGE. Gels were fixed and stained with Coomassie blue to confirm equal loading of GST-jun substrate and then exposed and quantitated on PhosphorImager cassettes (Storm; Molecular Dynamics, Sunnyvale, CA).

Data analysis. For TdT-mediated d-uridine triphosphate (UTP)-biotin nick-end labeling (TUNEL) assays, the total number of data points were derived as the sum of replicate wells (three or four) from at least three

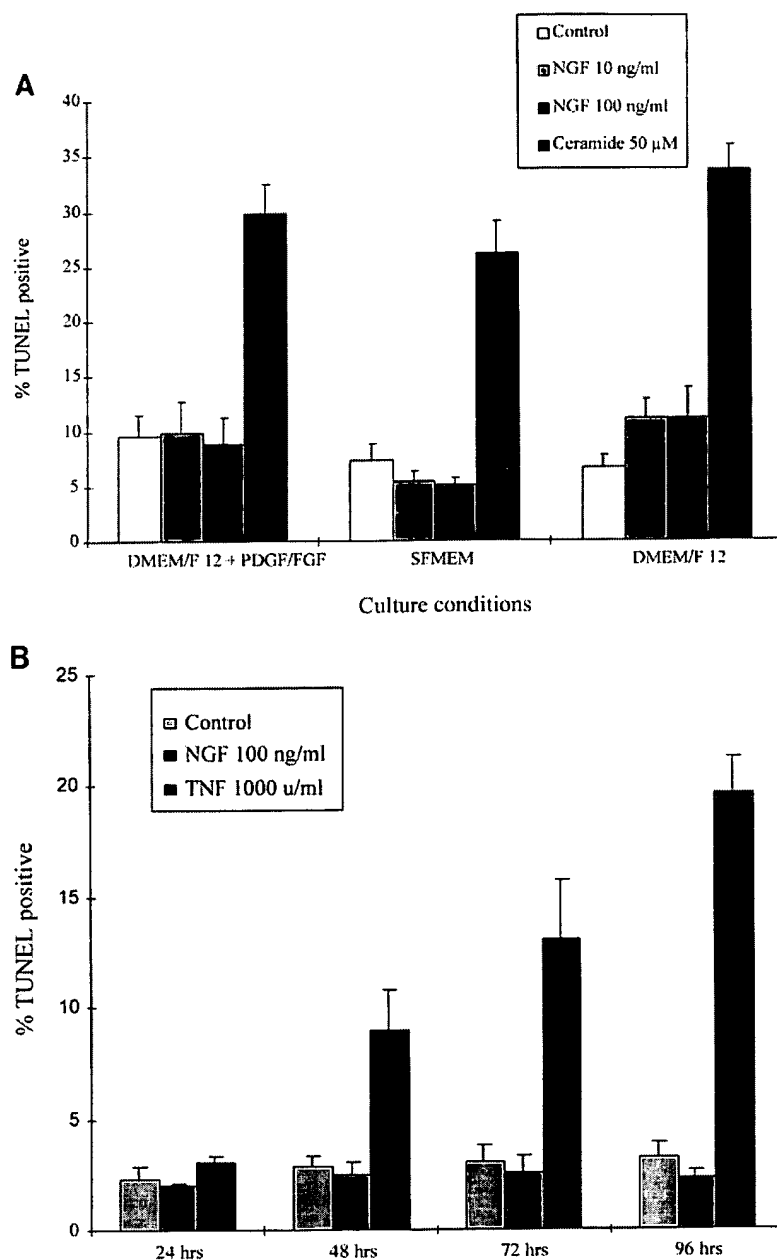


Figure 3. *A*, Effect of NGF on OLs in different culture conditions (DMEM/F12 with PDGF-AA/b-FGF, serum-free MEM, and DMEM/F12 without PDGF-AA/b-FGF) as measured by the TUNEL assay. Data indicate percent TUNEL-positive nuclei \pm SEM in nontreated or control cultures, in cultures with NGF at 10 ng/ml for 24 hr [$p > 0.05$, not significant (NS)], in cultures with NGF at 100 ng/ml for 24 hr ($p > 0.05$, NS), and in cultures with C2-ceramide at 50 μ M for 18 hr ($p < 0.01$) in each culture condition ($n = 4$). *B*, Percent TUNEL-positive nuclei in OLs cultured in serum-free MEM alone or exposed to either NGF at 100 ng/ml ($p > 0.05$, NS) or TNF- α at 1000 units/ml ($p < 0.01$) for 4 d.

separate experiments. Data collected from the TUNEL assays were analyzed for significance by the ordinary ANOVA test with Tukey-Kramer post tests if the p value was < 0.05 . For the JNK assay, significance was assessed using Student's t test.

RESULTS

Expression of p75^{NTR} but not trk A on adult human OLs

The morphological features of neural cells used in this study are illustrated in Figure 1, *A*, *D*, and *G*. Purity of the OLs in enriched cultures was estimated at 85–90% during the 2–3 week culture period. Contaminating cells included astrocytes and fibroblasts. OLs cultured in medium with b-FGF/PDGF-AA showed numerous processes. OLs grown in the different culture conditions (OLs in MEM with 5% FCS for 7 d then DMEM/F12 plus PDGF-AA/b-FGF, serum-free MEM, or DMEM/F12 only) were all O1 positive (Fig. 1*B*). P75^{NTR} expression was observed on cell bodies and

processes of OLs (Fig. 1*C*) under all three culture conditions. The absence of nonspecific immunoreactivity was verified by performing the immunocytochemistry protocol without primary antibody and by replacing primary antibody with isotypic control antibody. Contaminating cells with morphological characteristics of astrocytes or fibroblasts did not express p75^{NTR}. No p75^{NTR} expression could be detected on adult human microglia or fetal astrocytes in culture (Fig. 1*I*, *F*, respectively).

When the RT-PCR was performed on OL RNA using specific primers for p75^{NTR} and trkA, p75^{NTR} transcripts were detected in OLs from all three culture conditions (Fig. 2), consistent with the immunocytochemical data. Transcripts for trkA could not be detected in OLs from any of the conditions. Transcripts 2', 3'-cyclic nucleotide phosphodiesterase (CNPase), an OL-specific protein, were visualized (data not shown). Samples run without reverse transcriptase showed no contaminating bands.

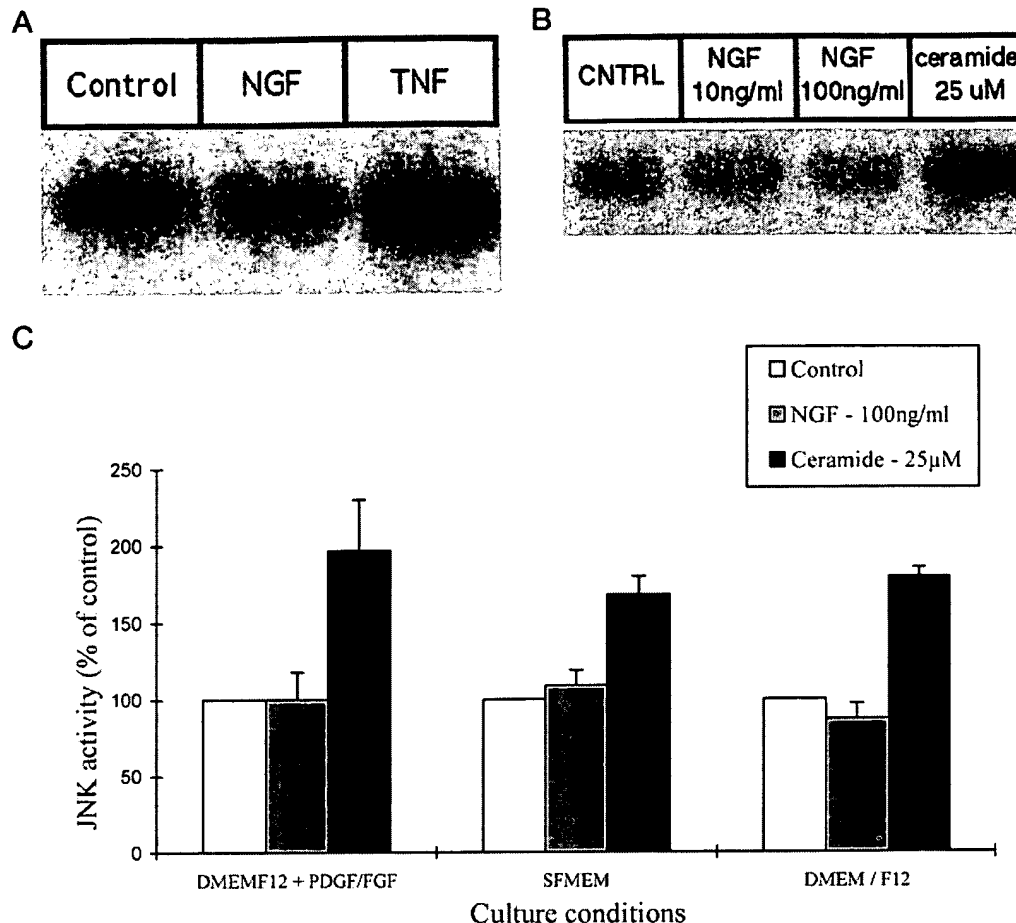


Figure 4. *A*, JNK activation in OLs treated with NGF or TNF- α . Adult human OLs were cultured in serum-free MEM; data are from untreated controls (lane 1) and cultures treated for 30 min with NGF at 100 ng/ml (lane 2) or with TNF- α at 10 ng/ml (lane 3). Only TNF- α significantly increased JNK activity as assessed by the level of phosphorylation of GST-jun. *B*, JNK activity in OLs treated with different concentrations of NGF and with C2-ceramide. Data are from OLs in serum-free MEM untreated (lane 1) or treated with NGF at 10 ng/ml for 3 hr (lane 2), with NGF at 100 ng/ml for 3 hr (lane 3), or with C2-ceramide at 25 μ M (lane 4). Significant JNK activation was observed with C2-ceramide only. *C*, JNK activity in response to NGF treatment of OLs cultured in different conditions. Adult human OLs were cultured in DMEM/F12 with PDGF-AA/b-FGF, serum-free MEM, or DMEM/F12 without PDGF-AA/b-FGF. Cells were then treated with NGF at 100 ng/ml or C2-ceramide at 25 μ M for 2 hr or left untreated (controls). JNK activity was quantified with a PhosphorImager. Values are expressed as the percent of the JNK activity of the control \pm SEM. Each bar represents the average of three different experiments. C2-ceramide induced significant JNK activation ($p < 0.05$) in each condition.

NGF does not cause significant apoptosis of OLs in different culture conditions

OLs in the different culture conditions described above were treated with 10 or 100 ng/ml of NGF for durations varying from 24 to 96 hr. TUNEL staining showed no significant apoptosis of the OLs in any of the three culture conditions with NGF at 10 ng/ml or 100 ng/ml at 24 hr (Fig. 3*A*). No increase in apoptosis was observed at any of the time points up to 96 hr. In contrast, TNF- α induced significant apoptosis of the OLs ($20 \pm 2\%$; $p < 0.01$) at 96 hr; Fig. 3*B*).

NGF treatment of OLs does not lead to significant JNK activation

JNK activity measured with a solid-phase kinase assay was not significantly increased when OLs in the different culture conditions were exposed to 100 ng/ml NGF at time points up to 3 hr (Fig. 4*A,B*). However, under identical conditions, both TNF- α (10 ng/ml) and C2-ceramide (25 μ M) were potent inducers of JNK (Fig. 4*A–C*).

NGF induces nuclear translocation of NF- κ B

Nuclear translocation of the p65 subunit of NF- κ B in response to 10 or 100 ng/ml NGF was detected by immunocytochemistry in significant numbers (26–47%) of OLs under all three culture conditions (Fig. 5*C,D*; Table 1) as compared with untreated (control) cultures (8–12%) (Fig. 5*A,B*; Table 1) and those treated with BDNF (7–11%) or NT-3 (7–8%) (Table 1). This effect was observed 3 hr after NGF treatment and was maintained for at least 6 hr. TNF- α induced strong nuclear staining at 30 min (data not shown).

DISCUSSION

In this study we show that OLs derived from human adult CNS express p75^{NTR} but not trkA under any of the culture conditions we have examined. In this regard, the adult human OLs differ from neonatal rat-derived OLs, because maintenance of rat-derived cells in b-FGF and PDGF-AA results in expression of trkA, which likely mediates a survival response by NGF (Cohen et al., 1996). The reasons for this difference in trkA expression is

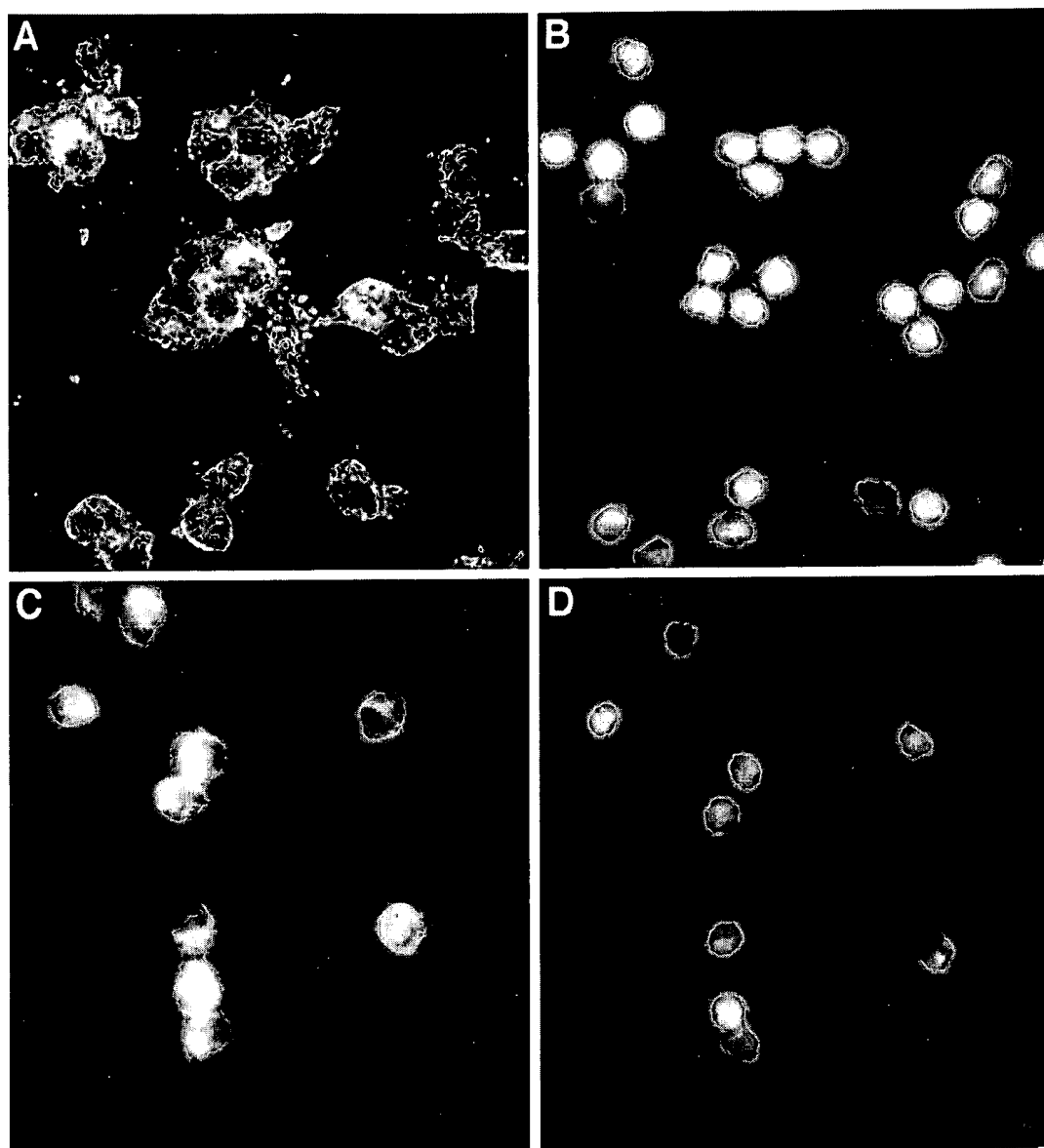


Figure 5. Nuclear translocation of NF- κ B is induced by NGF in OLs. *A*, Adult human OLs cultured in serum-free MEM; untreated controls with almost all OLs showing no nuclear staining for NF- κ B. *B*, Nuclear DNA of OLs in the same field as in *A* stained with Hoechst 33258. *C*, OLs in serum-free MEM treated with 100 ng/ml NGF for 3 hr and stained with a rabbit polyclonal antibody specific for the p65 subunit of NF- κ B. *D*, Nuclear staining of OLs in the same field as in *C* with Hoechst 33258. All microphotographs are at a magnification of 1000 \times .

Table 1. Nuclear translocation of NF- κ B is induced by NGF but not by BDNF or NT-3 in OLs

Culture condition	Untreated controls	BDNF (100 ng/ml)	NT-3 (100 ng/ml)	NGF (10 ng/ml)	NGF (100 ng/ml)
DMEM/F12	12 \pm 1	11 \pm 2	8 \pm 1	42 \pm 4	47 \pm 6
DMEM/F12 + PDGF/FGF	11 \pm 2	8 \pm 2	7 \pm 2	26 \pm 4	33 \pm 5
SF-MEM	8 \pm 2	7 \pm 3	8 \pm 2	26 \pm 2	29 \pm 4

Each treatment [NGF 10 and 100 ng/ml; BDNF (100 ng/ml); NT-3 (100 ng/ml) for 5 hr] was evaluated in replicate wells for each culture condition (DMEM/F12, DMEM/F12 with PDGF-AA/b-FGF, or serum-free MEM) on OLs derived from two different brain specimens ($n = 4$). A total of 75–100 cells were counted in each well. Data are presented as percent cells showing nuclear translocation \pm SEM.

unknown but could reflect a species-specific difference in gene expression as is seen in the expression of the CD59 receptor, a complement inhibitory protein, on human but not rodent OLs (Wing et al., 1992; Piddlesden and Morgan, 1993). It could also reflect the fact that the rat OLs used in previous studies were derived from neonatal animals, whereas the human OLs used in our studies have been postmitotic for a period of many years. In contrast, p75^{NTR} expression was detected in human OLs under all three culture conditions.

Our previous results have shown that TNF treatment of adult human OLs results in cellular apoptosis (D'Souza et al., 1995), indicating that a TNF-dependent apoptotic cascade is functional within these cells. In this study, we have compared the effects of TNF and NGF in our standard conditions as well as under the minimal culture conditions in which NGF treatment results in apoptosis of neonatal rat OLs. Treatment of these OLs with varying concentrations of NGF over a range of time periods resulted in no significant increase in the numbers of TUNEL-positive OLs, whereas significant injury was observed using TNF.

Several previous studies have examined the expression of NGF receptors and the effects of NGF on OLs, with considerable discord between the various results. Cohen et al. (1996) have found that neonatal rat-derived OLs maintained in b-FGF and PDGF-AA express both p75^{NTR} and trkA, and NGF promotes the survival of these cells. NGF-mediated proliferation and process extension have been reported for porcine OLs (Althaus et al., 1992). Using minimal media to maintain neonatal rat-derived immature OLs, Casaccia-Bonnet et al. (1996) found that rat OLs express p75^{NTR} but not trkA and that these cells underwent apoptosis in response to NGF. These differing effects of NGF might be explained if activation of trkA results in a dominant proliferation or survival signal that overrides an apoptotic signal derived from p75^{NTR}. However, our experiments show that human OLs do not express trkA under the culture conditions used either by Cohen et al. (1996) or Casaccia-Bonnet et al. (1996) but, nonetheless, fail to undergo NGF-mediated apoptosis and JNK activation under conditions in which activation of JNK and apoptosis do occur in response to TNF. This difference in responsiveness in the adult human OLs suggests that the signaling properties of p75^{NTR} show greater cell-type and developmental variability than do those of the apoptotic TNF-R1 and Fas receptors. Reasons for this are unclear but may include differential expression of appropriate p75^{NTR} signaling partners that remain unidentified.

Recently, p75^{NTR}-mediated signaling events, such as activation of NF- κ B transcriptional complex (Carter et al., 1996) and of the sphingomyelin–ceramide pathway (Dobrowsky et al., 1994), have been identified, and both of these signaling events are consistent with a role for p75^{NTR} in mediating injury responses within the CNS. However, activation of NF- κ B in several cell types blocks apoptotic cell death to a variety of insults, including TNF- α (Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996). Diverse functions other than apoptosis have been attributed to autonomous p75^{NTR} signaling in non-neuronal cells (Herrmann et al., 1993; Anton et al., 1994). The nuclear translocation of NF- κ B by NGF in human OLs may indicate a role for NF- κ B in preventing apoptosis, although its precise function remains to be determined.

Mature OLs within the CNS seem to express little, if any, p75^{NTR} under normal conditions (Yan and Johnson, 1988; Heuer et al., 1990; Yamamoto et al., 1993). Substantial amounts of p75^{NTR} were observed on apparently immature OLs in MS tissue

(Dowling et al., 1997). Furthermore, rats with experimental allergic encephalomyelitis, the animal model of MS, also have increased p75^{NTR} and NGF protein in their brains (De Simone et al., 1996). Rather than causing toxicity, NGF prevents autoimmune demyelination in marmosets (Diaz et al., 1997). β -Interferon, the approved therapy for MS, induces NGF production by astrocytes (Boutros et al., 1998). NGF and other neurotrophins continue to be considered for use as therapies in CNS neuronodegenerative disorders, emphasizing the need to define their effects on non-neuronal cells as a consequence of the therapy.

REFERENCES

- Althaus HH, Kloppner S, Schmidt-Schultz T, Schwartz P (1992) Nerve growth factor induces proliferation and enhances fiber regeneration in oligodendrocytes isolated from pig adult brain. *Neuroscience* 135:219–223.
- Anton ES, Weskamp G, Reichardt LF, Matthew WD (1994) Nerve growth factor and its low-affinity receptor promote Schwann cell proliferation. *Proc Natl Acad Sci USA* 91:2795–2799.
- Baker SJ, Reddy EP (1996) Transducers of life and death: TNF receptor superfamily and associated proteins. *Oncogene* 12:1–9.
- Barbacid M (1994) The trk family of neurotrophin receptors. *J Neurobiol* 25:1386–1403.
- Barker PA, Shooter EM (1994) Disruption of NGF binding to the low affinity neurotrophin receptor p75^{NTR} reduces NGF binding to trkA on PC12 cells. *Neuron* 13:203–215.
- Beg AA, Baltimore D (1996) An essential role for NF- κ B in preventing TNF- α -induced cell death. *Science* 274:782–784.
- Bonetti B, Raine CS (1997) Multiple sclerosis: oligodendrocytes display cell death-related molecules in situ but do not undergo apoptosis. *Ann Neurol* 42:74–84.
- Boutros T, Croze E, Yong VW (1997) Interferon- β is a potent promoter of nerve growth factor production by astrocytes. *J Neurochem* 69:939–946.
- Brück W, Schmied M, Suchanek G, Brück Y, Breitschopf H, Poser S, Piddlesden S, Lassmann H (1994) Oligodendrocytes in the early course of multiple sclerosis. *Ann Neurol* 35:65–73.
- Carter BD, Lewin GR (1997) Neurotrophins live and let die: does p75^{NTR} decide? *Neuron* 18:187–190.
- Carter BD, Kalschmidt C, Kalschmidt B, Offenhäuser, Böhm-Matthaei R, Baeuerle PA, Barde YA (1996) Selective activation of NF- κ B by nerve growth factor through the neurotrophin receptor p75. *Science* 272:542–545.
- Casaccia-Bonnet P, Carter BD, Dobrowsky RT, Chao MV (1996) Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. *Nature* 383:716–719.
- Chao MV (1994) The p75 neurotrophin receptor. *J Neurobiol* 25:1373–1385.
- Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159.
- Cohen R, Marmur R, Norton WT, Mehler MF, Kessler JA (1996) Nerve growth factor and neurotrophin-3 differentially regulate the proliferation and survival of developing rat brain oligodendrocytes. *J Neurosci* 16:6433–6442.
- D'Souza S, Alinauskas K, McRae E, Goodyer C, Antel JP (1995) Differential susceptibility of human CNS-derived cell populations to TNF-dependent and independent immune-mediated injury. *J Neurosci* 15:7293–7300.
- D'Souza SD, Bonetti B, Balasingam V, Cashman NR, Barker PA, Trout AB, Raine CS, Antel JP (1996) Multiple sclerosis: fas signalling in oligodendrocyte cell death. *J Exp Med* 184:2361–2370.
- D'Souza SD, Becher B, Trout AB, Antel JP (1998) Fas expression on human fetal astrocytes without susceptibility to fas-mediated cytotoxicity. *Neuroscience*, in press.
- De Simone R, Micera A, Tirassa P, Aloe L (1996) mRNA for NGF and p75 in the central nervous system of rats are affected by experimental allergic encephalomyelitis. *Neuropathol Appl Neurobiol* 22:54–59.
- Diaz VP, Bartke I, Unger J, Fisher S, Rosenberg D, Genain CP, Hauser SL (1997) Recombinant human nerve growth factor prevents autoimmune demyelination in marmosets. *Neurology* 48:S15.001.

- Dobrowsky RT, Werner MH, Castellino AM, Chao MV, Hannun YA (1994) Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. *Science* 265:1596-1599.
- Dopp JM, Mackenziegraham A, Otero GC, Merrill JE (1997) Differential expression, cytokine modulation and specific functions of type-1 and type-2 tumour necrosis factor receptors in rat glia. *J Neuroimmunol* 75:104-112.
- Dowling P, Sheng G, Raval S, Menonna J, Cook S, Husar W (1996) Involvement of the CD95 (APO-1/Fas) receptor/ligand system in multiple sclerosis brain. *J Exp Med* 184:1513-1518.
- Dowling PC, Husar W, Menonna J, Casaccia-Bonnel P, Chao M, Cook S (1997) Expression of p75 neurotrophin receptor in multiple sclerosis brain. *Neurology* 48:P06.083.
- Frade JM, Rodriguez-Tebar A, Barde YA (1996) Induction of cell death by endogenous nerve growth factor through its p75 receptor. *Nature* 383:166-168.
- Hantzopoulos PA, Suri C, Glass DJ, Goldfarb MP, Yancopoulos GD (1994) The low affinity NGF receptor p75 can collaborate with each of the trks to potentiate functional responses to the neurotrophins. *Neuron* 13:594-602.
- Herrmann JL, Menter DG, Hamed J, Marchetti D, Nakajima M, Nicolson GL (1993) Mediation of nerve growth factor-stimulated extracellular matrix invasion by the human melanoma. *Mol Biol Cell* 4:1205-1206.
- Heuer JG, Fatemie-Nainie S, Wheeler EF, Bothwell M (1990) Structure and developmental expression of the chicken NGF receptor. *Dev Biol* 137:287-304.
- Laudiero LB, Aloe L, Levi-Montalcini R, Butinelli C, Schilter D, Gillesse S, Otten U (1992) Multiple sclerosis patients express increased levels of the beta-nerve growth factor in cerebrospinal fluid. *Neurosci Lett* 147:9-12.
- Liu Z, Hsu H, Goeddel DV, Karin M (1996) Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF- κ B activation prevents cell death. *Cell* 87:565-576.
- Mahadeo D, Kaplan L, Chao MV, Hempstead BL (1994) High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors. *J Biol Chem* 269:6884-6891.
- Ozawa K, Suchanek G, Breitschopf H, Brück W, Budka H, Jellinger K, Lassmann H (1994) Patterns of oligodendroglia pathology in multiple sclerosis. *Brain* 117:1311-1322.
- Piddlesden SJ, Morgan BP (1993) Killing of rat glial cells by complement: deficiency of the rat analogue of CD59 is the cause of oligodendrocyte susceptibility to lysis. *J Neuroimmunol* 48:169-176.
- Raine CS (1990) Demyelinating diseases. In: *Textbook of neuropathology* (Davis RL, Robertson DM, eds), pp 535-620. Baltimore: Williams and Wilkins.
- Raine CS (1994) The Dale McFarlin Memorial Lecture: the immunology of the multiple sclerosis lesion. *Ann Neurol* 36:561-572.
- Raine CS, Scheinberg LC (1988) On the immunopathology of plaque development and repair in multiple sclerosis. *J Neuroimmunol* 20:189-201.
- Selmaj KW, Raine CS, Canella B, Brosnan CF (1991) Identification of lymphotoxin and tumour necrosis factor in multiple sclerosis lesions. *J Clin Invest* 87:949-954.
- Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM (1996) Suppression of TNF- α -induced apoptosis by NF- κ B. *Science* 274:787-789.
- Verdi JM, Birren SJ, Ibanez CF, Persson H, Kaplan DR, Benedetti M, Chao MV, Anderson DJ (1994) p75^{NGFR} regulates trk signal transduction and NGF-induced neuronal differentiation in MAH cells. *Neuron* 12:733-745.
- Westwick JK, Brenner DA (1995) Methods for analyzing c-jun kinase. *Methods Enzymol* 255:342-359.
- Wing MG, Zajicek J, Sceilly DJ, Compston DAS, Lachmann PJ (1992) Oligodendrocytes lack glycoprotein anchored proteins which protect them against complement lysis. Restoration of resistance to lysis by incorporation of CD59. *Immunology* 76:140-145.
- Yamamoto M, Sobue G, Mutoh T, Li M, Doyu M, Mitsuma T, Kimata K (1993) gene expression of high- (p140trk) and low-affinity nerve growth factor receptor (LNGFR) in the adult and aged human peripheral nervous system. *Neurosci Lett* 158:39-43.
- Yan Q, Johnson JEM (1988) An immunohistochemical study of nerve growth factor receptor in developing rats. *J Neurosci* 8:3481-3498.
- Yong VW, Antel JP (1992) Culture of glial cells from human biopsies. In: *Protocols for neural cell culture* (Richardson A, Fedoroff S, eds), pp 81-86. St. Louis: Humana.

BRIEF REVIEWS

Of Mice and Not Men: Differences between Mouse and Human Immunology

Javier Mestas and Christopher C. W. Hughes¹

Mice are the experimental tool of choice for the majority of immunologists and the study of their immune responses has yielded tremendous insight into the workings of the human immune system. However, as 65 million years of evolution might suggest, there are significant differences. Here we outline known discrepancies in both innate and adaptive immunity, including: balance of leukocyte subsets, defensins, Toll receptors, inducible NO synthase, the NK inhibitory receptor families Ly49 and KIR, FcR, Ig subsets, the B cell (BLNK, Btk, and $\lambda 5$) and T cell (ZAP70 and common γ -chain) signaling pathway components, Thy-1, $\gamma\delta$ T cells, cytokines and cytokine receptors, Th1/Th2 differentiation, costimulatory molecule expression and function, Ag-presenting function of endothelial cells, and chemokine and chemokine receptor expression. We also provide examples, such as multiple sclerosis and delayed-type hypersensitivity, where complex multicomponent processes differ. Such differences should be taken into account when using mice as preclinical models of human disease. The Journal of Immunology, 2004, 172: 2731–2738.

Mice are the mainstay of in vivo immunological experimentation and in many respects they mirror human biology remarkably well. This conservation of function is reflected in recent reports on the sequencing of both the human and mice genomes, which reveal that to date only 300 or so genes appear to be unique to one species or the other (1). Despite this conservation there exist significant differences between mice and humans in immune system development, activation, and response to challenge, in both the innate and adaptive arms. Such differences should not be surprising as the two species diverged somewhere between 65 and 75 million years ago, differ hugely in both size and lifespan, and have evolved in quite different ecological niches where widely different pathogenic challenges need to be met—after all, most of us do not live with our heads a half-inch off the ground. However, because there are so many parallels there has been a tendency to ignore differences and in many cases, perhaps, make the assumption that what is true in mice—in vivo veritas—is neces-

sarily true in humans. By making such assumptions we run the risk of overlooking aspects of human immunology that do not occur, or cannot be modeled, in mice. Included in this subset will be differences that may preclude a successful preclinical trial in mice becoming a successful clinical trial in human.

In this review our aim is not to suggest that the mouse is an invalid model system for human biology. Clearly, with so many paradigms that translate well between the species, and with the relative ease with which mice can now be genetically manipulated, mouse models will continue to provide important information for many years to come. Rather, our aim is to sound a word of caution. As therapies for human diseases become ever more sophisticated and specifically targeted, it becomes increasingly important to understand the potential limitations of extrapolating data from mice to humans. The literature is littered with examples of therapies that work well in mice but fail to provide similar efficacy in humans (2–7). By focusing on some known differences between mouse and human immunology we hope to spur interest in this area and encourage others to note differences where they occur.

Structure and general characteristics

The overall structure of the immune system in mice and humans is quite similar. As this topic has been recently reviewed in depth (8), we will not go into great detail here. One difference worth noting is that whereas mice have significant bronchus-associated lymphoid tissue, this is largely absent in healthy humans (9), possibly reflecting a higher breathable Ag load for animals living so much closer to the ground.

The balance of lymphocytes and neutrophils in adult animals is quite different: human blood is neutrophil rich (50–70% neutrophils, 30–50% lymphocytes) whereas mouse blood has a strong preponderance of lymphocytes (75–90% lymphocytes, 10–25% neutrophils) (10). It is not clear what, if any, functional consequence this shift toward neutrophil-rich blood in humans has had.

Tyrosine kinase receptor expression on putative hemopoietic stem cells (HSC)² shows a reciprocal pattern, with mouse HSC being predominantly *c-kit*^{high}, *flt-3*[−], whereas human HSC are predominantly *c-kit*^{low}, *flt-3*⁺ (11).

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² Abbreviations used in this paper: HSC, hemopoietic stem cells; iNOS, inducible NO synthase; γ_c , common γ -chain; DETC, dendritic epidermal T cells; MS, multiple sclerosis; DTH, delayed-type hypersensitivity; EC, endothelial cells.

Innate immunity

One of the first lines of defense in higher organisms, and often the only defense in lower animals, is the growing family of antimicrobial peptides, and in particular the defensins. These are important in mucosal defense in the gut and in epithelial defense in skin and elsewhere (12, 13). Neutrophils are a rich source of leukocyte defensins in humans, but defensins are not expressed by neutrophils in mice (14). In contrast, Paneth cells, which are present in the crypts of the small intestine, express >20 defensins (cryptidins) in mice but only two in human, likely reflecting different evolutionary pressures related to microorganism exposure through food intake. There are also differences in processing of defensins (Table I).

The last few years have seen a renewed focus on the field of innate immunology, spurred in large part by identification of the Toll-like family of receptors—the TLRs (15). This field is still relatively young and so far a limited number of differences have been noted between mice and humans (Table I).

There has been considerable controversy as to whether human macrophages express NO. Expression of functional inducible NO synthase (iNOS; NOS2) in mouse macrophages has been clearly demonstrated and iNOS mRNA is readily induced by IFN- γ and LPS (16). However, these same inflammatory mediators have failed to show consistent effects on human macrophages, hence the confusion. Recent work suggests that other mediators, such as IFN- $\alpha\beta$, IL-4 plus anti-CD23, and various chemokines, are actually far more efficient in inducing iNOS in human macrophages (17). However, the controversy is not dead yet (18).

Using different strains of mice a susceptibility locus for CMV infection, *cmv1*, was identified and later shown to encode the Ly49 family of proteins (19). There are at least 14 members and most are expressed on NK and NKT cells, where the majority act as NK inhibitory receptors for MHC I molecules. The Ly49 family is absent in humans, who use the KIR family as NK inhibitory receptors (20). KIR proteins are highly diverged from the Ly49 family and have Ig rather than C-type lectin domains in their extracellular domain; however, similarly to Ly49 they also recognize MHC class I. The ligands for mouse and human NKG2D differ: in humans, NKG2D binds the polymorphic MHC class I-like molecules MHC-I chain-related A, MHC-I chain-related B, and the UL16 binding protein family, whereas in mouse NKG2D binds to H-60 and Rae1 β . The significance of these differences to CMV infection and to NK biology in general have not been determined.

Adaptive immunity

FcR represent a link between the adaptive immune system, which generates Ab, and the innate immune system, which can respond to Ab-Ag complexes through capture by FcR expressed on macrophages, neutrophils, eosinophils, mast cells, and dendritic cells. There are several differences in FcR expression between mice and humans. In humans, Fc α RI (CD89) is an important IgA receptor expressed by neutrophils, eosinophils, monocytes/macrophages, dendritic cells, and Kupffer cells (21). Mice lack Fc α RI and presumably use alternative receptors, such as Fc α / μ R, the transferrin receptor (CD71) and polymeric IgR, which also binds IgM. Humans also express two IgG receptors not found in mice: Fc γ RIIA and Fc γ RIIC are closely related single-chain FcR, each of which has a single ITAM motif in the intracellular domain. In contrast, most

other FcR associate with ITAM-containing signal transduction subunits (22).

In addition to differences in FcR there are well-known differences in expression of Ig isotypes between mice and humans, and direct correlations between subtypes within classes in each species are hard to make. Mice make IgA, IgD, IgE, IgM, and four subtypes of IgG: IgG1, IgG2a, IgG2b, and IgG3. Interestingly, in the C57BL/6, C57BL/10, SJL, and NOD strains of mice there is no expression of IgG2a, instead these mice express the novel IgG2c (23). Humans in contrast express two subtypes of IgA—IgA1 and IgA2—along with single forms of IgD, IgE, and IgM. In humans there are also four subtypes of IgG: IgG1, IgG2, IgG3, and IgG4; however, these are not direct homologues of the mouse proteins. While different subtypes have differing abilities to bind FcR or fix complement, the differences between mice and humans are not considered significant. In contrast, there are differences in class switching: in mice, IL-4 induces IgG1 and IgE, whereas in humans, IL-4 induces switching to IgG4 and IgE. In contrast, IL-13 has no effect on mouse B cells but induces switching to IgE in humans (24).

There are some interesting differences in B cell development that relate to the roles of several signaling molecules. BLNK (Src homology-2 domain containing leukocyte-specific phosphoprotein-65) is an adapter protein that is rapidly phosphorylated by Syk after cross-linking of the B cell Ag receptor. It then serves as a scaffold for downstream signaling components such as Grb2, Vav, Nck, and PLC- γ . B cell development in mice lacking BLNK is blocked at the pro-B to pre-B transition, resulting in low numbers of IgM⁺ B cells, but no mature IgM^{low}IgD^{high} B cells, appearing in the periphery (25). A naturally occurring mutation in the human BLNK protein has been identified that results in a splicing defect preventing protein expression. In this patient there was also a block in the pro-B to pre-B transition; however, there was also a complete absence of B cells in the periphery, suggesting a more severe block in human B cell development than in mice (26).

Similarly discrepant phenotypes have been noted in mice lacking functional BCR-associated tyrosine kinase Btk (27) and in mice lacking λ 5 (28), the L chain component of the pre-BCR (Table I). Differences in mature B cells between mice and humans were recently reviewed (29), and include mutually exclusive expression of CD5 and CD23 on mouse but not human B cell subsets, and CD38 expression on human, but not mouse, plasma cells.

The discrepant phenotypes discussed above for BLNK, Btk, and λ 5 should be treated with some caution as the human diseases usually arise due to mutations in the relevant genes rather than deletions of whole exons as seen in the mouse knockout models. In some cases, however, identical mutations have been found, or created, in mice and the discrepant phenotype remains. This is the case for human XLA and mouse XID, which both involve Btk (30, 31).

The development and regulation of T cells also differs between mice and humans. Thy-1 is a GPI-linked Ig superfamily molecule of unknown function. It is expressed on thymocytes and peripheral T cells in mice and has been widely used as a T cell marker in the thymus. In humans, however, it is only expressed on neurons. The basis of this tissue specificity is suggested to be the presence or absence of an Ets-1 binding site in the third intron of the gene (32).

Table I. Summary of some known immunological differences between mouse and human

	Mouse	Human	Notes	Refs.
Hematopoiesis in spleen	Active into adulthood	Ends before birth		9
Presence of BALT	Significant	Largely absent in healthy tissue		10
Neutrophils in periph. blood	10–25%	50–70%		10
Lymphocytes in periph. blood	75–90%	30–50%		11
Hematopoietic stem cells	<i>c-kit^{high}, flt-3⁻</i>	<i>c-kit^{low}, flt-3⁺</i>		88
TLR2 expression on PBL	Low (induced on many cells including T cells)	Constitutive (but not on T cells)	Binds lipopeptides	88, 89
TLR3	Expressed on DC, Mac. Induced by LPS	Expressed by DC. No LPS induction	Binds dsRNA	90, 91
TLR9	Expressed on all myeloid cells, plasmacytoid DC and B cells	Expressed only on B cells, plasmacytoid DC and N	Binds CpG	92
TLR10	Pseudogene	Widely expressed		93
Sialic acid Neu5GC expression	Widespread	Absent	Binds pathogens	94
CD33	Expressed on granulocytes	Expressed on monocytes	Binds sialic acids	14
Leukocyte defensins	Absent	Present	neutrophils	94, 95
Paneth cell defensins	Processed by MMP7. Stored pre-processed	Stored as pro-form. Processed by trypsin		13
Paneth cell defensins	At least 20	Two		17
Macrophage NO	Induced by IFN- γ and LPS	Induced by IFN- α/β , IL-4 ⁺ anti-CD23		96
CD4 on macrophages	Absent	Present		40
Predominant T cells in skin and mucosa	γ/δ TCR (dendritic epidermal T cells—DETC)	α/β TCR		97
γ/δ T cells respond to phospho-antigens	No	Yes		41
CD1 genes	CD1d	CD1a,b,c,d		20
NK inhibitory Rs for MHC I	Ly49 family (except Ly49D and H)	KIR		98
NKG2D ligands	H-60, Rae1 β	MIC A, MIC B, ULBP	NK activating Rs	99
fMLP receptor affinity	Low	High		21
Fc α RI	Absent	Present		22
Fc γ RIIA, C	Absent	Present		21
Serum IgA	Mostly polymeric	Mostly monomeric		23
Ig classes	IgA, IgD, IgE, IgG1, IgG2a*, IgG2b, IgG3, IgM * absent in C57BL/6, /10, SJL and NOD mice, which have IgG2c	IgA1, IgA2, IgD, IgE, IgG1, IgG2, IgG3, IgG4, IgM		100
Ig CDR-H3 region	Shorter, less diverse	Longer, more diverse		25, 26
BLNK deficiency	IgM ^{high} B cells in periphery	No peripheral B cells		28
Btk deficiency	Normal pre-B and immature B	Blocks pro-B to pre-B transition		28
λ 5 deficiency	"leaky" block at pro-B to pre-B transition	Blocks pro-B to pre-B transition		29
CD38 expression on B cells	Low on GC B cells, off in plasma cells	High on GC B cells and plasma cells		29
B cell CD5 and CD23 expression	Mutually exclusive	Co-expression		24
IL-13 effect on B cells	None	Induces switch to IgE		32
Thy 1 expression	Thymocytes, peripheral T cells	Absent from all T cells, expressed on neurons		33, 34
Effect of γ_c deficiency	Loss of T, NK, and B cells	Loss of T, NK, but B cell numbers normal		31
Effect of Jak3 deficiency	Phenocopies γ_c deficiency	Phenocopies γ_c deficiency		35, 36
Effect of IL-7R deficiency	Blocks T and B cell development	Only blocks T cell development		37, 38
ZAP70 deficiency	No CD4 ⁺ or CD8 ⁺ T cells	No CD8 ⁺ T but many nonfunctional CD4 ⁺	Related to syk level?	62, 63
Caspase 8 deficiency	Embryonic lethal	Viable—immunodeficiency		62
Caspase 10	Absent	Present		44
IFN- α promotes Th1 differentiation	No	Yes	Mutant stat2 in mice	51
Th expression of IL-10	Th2	Th1 and Th2		54
IL-4 and IFN- γ expression by cultured Th	Either/or	Sometimes both		55–57
CD28 expression on T cells	On 100% of CD4 ⁺ and CD8 ⁺	On 80% of CD4 ⁺ , 50% of CD8 ⁺		101–2
ICOS deficiency	Normal B cell numbers and function, normal IgM levels	B cells immature and severely reduced in number, low IgM	Possibly age-related	103–4
B7-H3 effects on T cells	Inhibits activation	Promotes activation		58
ICAM3	Absent	Present	DC-SIGN ligand	105
P-selectin promoter	Activated by TNF and LPS	Unresponsive to inflammation		59–61
GlyCAM	Present	Absent		64, 65
MHC II expression on T cells	Absent	Present	Regulates Ca flux	106
Kv1.3 K ⁺ channel on T cells	Absent	Present	Regulates migration?	43
MUC1 on T cells	Absent	Present	In CTL	
Granulysin	Absent	Present		

(Table continues)

Table 1. *Continues*

Mouse		Human	Notes	Refs.
CXCR1	Absent	Present		66, 67
IL-8, NAP-2, ITAC, MCP-4, HCC-1, HCC-2, MPIF-1, PARC, eotaxin-2/3	Absent	Present	Chemokines	66, 67
MRP-1/2, lungkine, MCP-5	Present	Absent	Chemokines	66, 67
IFN- γ effects in demyelinating disease	Protective in EAE	Exacerbates MS		4, 69–70
DTH lesions	Neutrophil-rich	Lymphocyte-rich		73, 74
Constitutive MHC II on EC	Absent	Present		80
EC present Ag to CD4+ T	No	Yes	Memory T only	75–77
CD58 (LFA-3)	Absent	Present	CD2 ligand	82
T cell dependence on CD2-ligand interactions	Low	High		82
CD2-ligand interaction	Lower affinity, with CD48	Higher affinity, with CD58		82
CD40 on EC	Absent	Present		83, 84
Vascularized grafts tolerogenic?	Yes	No		5
Microchimerism induces graft tolerance?	High success rate	Low success (expts. in non-human primates)		7
Passenger leukocytes	Account for graft immunogenicity	Do not account for graft immunogenicity		6

Similar to the development of B cells, mutation of key signaling molecules in T cells has markedly different effects in mice and humans. Several cytokine receptors, including those for IL-2, IL-4, IL-7, IL-9, and IL-15, share a common signaling chain called common γ chain (γ_c). Perhaps not surprisingly, deletion or mutation of this gene, which is on the X chromosome, results in severe immunological defects. Interestingly, these differ between human and mouse XSCID (33, 34). Numerous mutations have been identified in the human γ_c gene that inhibit function, and in most of these cases the result is a dramatic decrease in the number of T cells and NK cells. However, B cell development is normal, although function is impaired, likely due to the lack of T cell help. In marked contrast, B cell numbers are greatly diminished in γ_c -null mice. Given that IL-7R deficiency in mice blocks both T and B cell development (35), but only blocks T cell development in humans (36), it is likely that B cell development in humans is independent of IL-7. The major signal transducer for γ_c is JAK3 and mutation of this gene phenocopies the γ_c mutation in both mice and humans; that is, a lack of T and NK cells in human with the addition of a severe B cell defect in mice (31).

Interesting differences have also been noted in ZAP70-deficient mice and humans. ZAP70 is essential for TCR signaling in both developing and mature T cells, and compromised signaling results in SCID. In humans the defect results in normal numbers of CD4⁺ T cells and absent CD8⁺ T cells. However, the CD4⁺ T cells are nonfunctional. In contrast, an identical mutation introduced into the mouse ZAP70 results in a block in differentiation of both T cell subsets at the double-positive stage (37). It has been suggested that the "leakiness" of the human mutant is due to incomplete down-regulation of the protein tyrosine kinase Syk in human thymocytes, compared with mouse thymocytes (38).

The study of γ/δ T cells has revealed a number of significant differences between mice and humans. T cells expressing γ/δ TCR are found in all organisms that have α/β receptors and yet their function is still largely an enigma (39). Mouse skin contains a large fraction of cells bearing a TCR encoded by a single V γ and V δ gene. These V γ 5-V δ 1 T cells appear to be oligoclonal, reside in the epidermis, and are known as dendritic epidermal T cells (DETC). DETC represent the predominant T

cell in mouse skin, whereas cells bearing α/β receptors predominate in human skin and are found mostly in the dermis. Indeed, a cell with DETC characteristics has not been identified in humans (40). Human but not mouse γ/δ T cells have been suggested to recognize Ag presented by CD1 molecules—in particular CD1b (41). Interestingly, of the five CD1 molecules found in humans (designated CD1a, b, c, d, and e), only CD1d is expressed in mice (41). Similarly to γ/δ T cells the CD1 family of molecules has been implicated in the pathogenesis of tuberculosis, but their precise role has yet to be defined (42, 43). The differing expression of CD1 genes between mice and humans may well turn out to impact activation of both α/β and γ/δ T cells in tuberculosis, as both subsets can recognize a variety of Ags presented by CD1 molecules.

An often critical component of adaptive immunity is the skewing of T cell differentiation toward Th1 or Th2 phenotypes and this process represents another area of interaction between the innate and adaptive arms of immunity. In humans, the type I IFN, IFN- α , is secreted by several cell types in response to viral infection, including macrophages, and acts on T cells to induce Th1 development. This process is dependent upon STAT4 activation, and its recruitment to the IFN- α receptor by STAT2. In mice, however, IFN- α fails to induce Th1 cells and does not activate STAT4 (44).

The existence of polarized T cell populations was first demonstrated by Mosmann and colleagues (45) and since then has become a guiding principle for T cell activation. While polarization is relatively easy to observe in mice the paradigm has never been as clear-cut in the human system. Th1 and Th2 cells can certainly be found in human disease (46, 47); however, there is a growing recognition that in many diseases clear distinctions cannot be made and that T cells of both persuasions can often be generated simultaneously (48–50). For example, in mice, IL-10 is considered to be a Th2 cytokine, whereas in humans both Th1 and Th2 cells can make IL-10 (51). The response of mice and humans to schistosomiasis is remarkably different. Epidemiological data suggest that a Th2 response involving eosinophils and IgE may be key to combating infection in humans (52), whereas in mice effector cell activation by IFN- γ , a Th1 response, is essential for clearance of the parasite (53).

To become fully activated T cells require both a primary, Ag-dependent signal, and a second, Ag-independent or costimulatory signal. One of the best characterized costimulatory receptors is CD28, which is expressed by close to 100% of mouse CD4⁺ and CD8⁺ T cells. In contrast, only 80% of human CD4⁺ and 50% of human CD8⁺ T cells express CD28 (54), perhaps accounting for the remarkable efficacy of CTLA-4Ig in blocking T cell activation in mice. It will be interesting to see if expression of the CD28-related costimulatory molecule ICOS segregates with CD28⁺ T cells in humans. The recent report on the identification of a human ICOS deficiency pointed to a further difference between costimulation in mice and humans. Whereas in mice the loss of ICOS does not affect either the number of mature B cells, their maturation status or their secretion of IgM (55, 56), the loss of ICOS in humans results in a severe reduction in B cell number, maturation status and secretion of IgM (57). Given the critical role of T cell CD40L in T-B interactions it would be interesting to know what the level of CD40L expression was on this patient's T cells and whether expression of this molecule is dependent upon ICOS signaling in humans. Two novel members of the B7 family of costimulatory molecules, B7-H3 and DC-SIGN, have recently also been suggested to have different roles in mice and humans (Table I).

P-selectin is constitutively expressed by endothelial cells (EC) and mediates leukocyte rolling by interactions with specific sugar residues carried by mucins. Interestingly, murine P-selectin can be strongly up-regulated by inflammatory mediators such as TNF and LPS, whereas the human gene is nonresponsive (58). It is interesting to speculate as to whether E-selectin in humans, which is strongly up-regulated by TNF, is the more important selectin on human EC for mediating leukocyte rolling.

Once activated, human T cells express MHC class II molecules whereas murine T cells do not. It has been suggested that human T cells can capture, process, and present Ag and that they express B7 and may therefore help to amplify an ongoing immune response (59, 60). In contrast, Ag presentation by T cells may also promote T cell anergy (61) or activation-induced cell death. It is not clear why this function is nonessential in mice, but it is an attractive hypothesis that it may relate to T cell homeostasis and the requirement in humans for maintaining, in a limited compartment, a greater diversity of memory T cells for a considerably longer period of time than is required in mice. T cell homeostasis requires programmed cell death (apoptosis) of unwanted cells. Caspase 8 and caspase 10 are downstream of death receptors in humans and overlap in some of their functions (62). Mice lack caspase 10 and the deletion of caspase 8 is embryonic lethal. Lack of caspase 8 in humans results in immunodeficiency, suggesting a role for this effector in lymphocyte activation as well as death (63). Greater redundancy in death receptor regulators in humans may relate to the longer lifespan and associated increased risk of developing cancer.

A critical step in activation of a T cell is the generation of a sustained calcium flux. In human T cells the inward flow of calcium ions is balanced by an outward flow of K⁺, mediated in large part by the Kv1.3 K⁺ channel. Inhibitors of this channel very specifically block T cell activation *in vitro* and are being pursued as novel immunosuppressive agents (64). However, *in vivo* evidence to support such a function is missing as mouse T cells do not express this channel (65).

The movement of immune cells into and through tissues is coordinated by a huge array of chemokines and chemokine receptors and, not surprisingly, differences have emerged between the murine and human systems. While it is still too early to say definitively what such differences may mean, as there appears to be considerable redundancy built into the system, it is worth noting what is currently known. CXCR1 is present in humans but not in mice (66). The chemokines IL-8 (CXCL8), neutrophil-activating peptide-2 (CXCL7), IFN-inducible T cell α -chemoattractant (CXCL11), monocyte chemoattractant protein (MCP)-4 (CCL13), HCC-1 (CCL14), hemofiltrate CC chemokines-2 (CCL15), pulmonary and activation-regulated chemokine (CCL18), myeloid progenitor inhibitory factor-1 (CCL23), and eotaxin-2/3 (CCL24/CCL26) have all been identified in humans but not in mice. Conversely, CCL6, CCL9, lungkine (CXCL15), and MCP-5 (CCL12) have been identified in mice but not humans (66, 67).

Differences in immune system biology

Multiple sclerosis (MS) provides a fine example of both differences and similarities between mouse and human immunology. MS is a multifactorial disease that appears to have a large autoimmune component (68). Experimental autoimmune (allergic) encephalomyelitis is a widely used model for MS that mimics the demyelination seen in central and peripheral nerves in MS. Several studies have indicated that IFN- γ is protective in experimental autoimmune (allergic) encephalomyelitis as neutralizing Abs exacerbate disease, potentially by blocking induction/activation of suppressor activity (69, 70). It was surprising, therefore that clinical trials were not successful; indeed they were stopped because treatment with IFN- γ was found to exacerbate disease (4). In contrast, studies in mice suggested that blocking VLA-4 ($\alpha_4\beta_1$ integrin)-VCAM-1 interaction might help in MS (71) and this has indeed carried through successfully into human trials (72). These studies highlight how caution is required when extrapolating results from mouse studies to the clinic, but suggest that mouse models can successfully predict some therapies for human disease.

An interesting difference exists in the appearance of delayed-type hypersensitivity (DTH) reactions in mice and humans. In humans, around four hours after Ag challenge neutrophils can be seen forming a "cuff" around the venules. This is followed by a dramatic influx of mononuclear cells, such that by 24–48 h the lesion is mostly mononuclear with a mix of T cells and macrophages (73). Paradoxically, in mice where the peripheral blood has a relative paucity of neutrophils compared with humans, the DTH response tends to be more neutrophil rich (74). In addition, elicitation of murine DTH requires much higher concentrations of Ag than in humans.

There is now considerable evidence that human EC can present Ag to resting memory CD4⁺ and CD8⁺ T cells (75–77), whereas in mice, CD8⁺ T cells can be activated by EC (78), but CD4⁺ T cells cannot (B. Rosengard, personal communication). As CD4⁺ T cell-mediated activation of macrophages is thought to drive human DTH responses the suggestion has arisen that in humans, Ag transport to lymph nodes by Langerhans cells may not be necessary as EC may trigger the recall response at the site of challenge. A teleological argument can be made for the need to present Ag locally in humans but not necessarily in mice. It has been estimated that once a cell enters the lymphatics in humans it takes ~24 h to return to the

circulation if it is not retained in a node (79). Based on the higher cardiac output of mice as a proportion of their total blood volume compared with humans (5–10 ml/min, 2 ml total volume in mice; 5 L/min, 5 liter total volume in humans) it is reasonable to suppose that return of lymph is at least as fast in mice as it is in humans. Then it becomes a matter of scale. We calculate that an Ag traveling from toe to an inguinal lymph node in the groin should take ~12 h in humans and 20 min in mice. As the human DTH response begins around 4 h after secondary Ag challenge, it is possible that triggering of recall responses may occur by different mechanisms in mice and humans, involving draining of Ag to lymph nodes in mice, compared with local Ag presentation in humans.

Both human and mouse EC express MHC class I. Most human EC in vivo also constitutively express MHC class II molecules, whereas mouse EC do not (80). Thus, human EC can present Ag to CD4⁺ T cells, as well as to CD8⁺ T cells. A major costimulatory molecule on human EC is CD58 (LFA-3), a ligand for CD2 (81). Mice do not have the gene for CD58, which arose by CD2 gene duplication after the two lineages split. In mice the CD2 ligand is CD48; however, the distribution of this molecule differs from that of CD58 in humans, and the two-dimensional affinity for the mouse CD2-CD48 interaction is 40- to 50-fold lower than that for human CD2-CD58 interactions (82). In addition, gene deletion and Ab blocking studies have shown that mouse T cell activation is much less dependent on CD2 interactions than is the case for human T cells. Human EC also express CD40 and the ICOS ligand GL-50, whereas murine EC do not (83, 84).

The Ag presenting ability of human EC may have significant consequences for transplantation. For example, in many rodent models vascularized grafts are tolerizing, whereas such grafts are rapidly rejected in humans (5). Numerous studies have shown that purging mouse tissues of CD45⁺ cells before transplantation dramatically extends the life of the graft, sometimes even inducing tolerance. In sharp contrast, purging human tissues of CD45⁺ cells provides no benefit as the grafts are still rapidly rejected (6). In addition, the establishment of microchimerism in mice has been quite successful in inducing tolerance, whereas this has not been the case in humans (7). The implication of these findings is that there are major differences between mice and humans in their responses to grafted tissue, and that this may relate to the Ag-presenting ability of human, but not murine, EC.

Natural selection and the immune system

Most, if not all, of the differences we have noted between mouse and human immunology have likely become fixed during the 65 million years since our divergence because they provide some selective advantage. In all likelihood these adaptations are in response to new pathologic challenges from microorganisms, which have very short generation times and often have high mutation rates (85). In consequence, mammalian MHC molecules and NK cell inhibitory receptors have also evolved rapidly (9, 86). It should also be noted that some changes may be fixed primarily as a result of the nonimmune role of that gene—reiterative use of genes is a well recognized phenomenon during development, a good example being the important nonimmunological role of VCAM in chorioallantoic fusion and placentation (87). Thus, both the immune system as a whole, and

some of its individual components (B and T cell repertoires) are shaped by natural selection.

Mice evolved in a quite different environment to humans and have been exposed to different Ags and their immune systems might therefore be expected to have evolved in subtly different ways. Mice not only live in different ecological niches, they are also much smaller and have significantly shorter lifespans. These are not trivial differences—as noted above, leukocyte transit times may be quite different in mice and humans, and a larger, broader repertoire of B and T cells must be maintained for many years in humans (up to 50 mouse lifetimes). Thus many changes may be to accommodate increased size of the organism, to regulate larger and more diverse pools of Ag-specific cells, and to provide greater checks and balances to combat the increased somatic mutation load that longer-lived animals necessarily carry.

Summary

While it is hard to draw global conclusions about the significance of differences between mouse and human immunology, it is worth considering the possibility that any given response in a mouse may not occur in precisely the same way in humans. While caution in interpreting preclinical data obtained in mice is clearly warranted, we believe that with these caveats in mind, mice will continue to be the premiere in vivo model for human immunology and will be absolutely essential for continued progress in our understanding of immune system function in health and disease.

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References

1. Waterston, R. H., K. Lindblad-Toh, E. Birney, J. Rogers, J. F. Abril, P. Agarwal, R. Agarwala, R. Ainscough, M. Alexandersson, P. An, et al. 2002. Initial sequencing and comparative analysis of the mouse genome. *Nature* 420:520.
2. Shepherd, F. A., and S. S. Sridhar. 2003. Angiogenesis inhibitors under study for the treatment of lung cancer. *Lung Cancer* 41(Suppl. 1):S63.
3. Oehler, M. K., and R. Bicknell. 2000. The promise of anti-angiogenic cancer therapy. *Br. J. Cancer* 82:749.
4. Panitch, H. S., R. L. Hirsch, A. S. Haley, and K. P. Johnson. 1987. Exacerbations of multiple sclerosis in patients treated with γ interferon. *Lancet* 1:893.
5. Sykes, M. 2001. Mixed chimerism and transplant tolerance. *Immunity* 14:417.
6. Wood, K. J. 2003. Passenger leukocytes and microchimerism: what role in tolerance induction? *Transplantation* 75:175.
7. Monaco, A. P. 2003. Chimerism in organ transplantation: conflicting experiments and clinical observations. *Transplantation* 75:135.
8. Haley, P. J. 2003. Species differences in the structure and function of the immune system. *Toxicology* 188:49.
9. Pabst, R., and I. Gehrke. 1990. Is the bronchus-associated lymphoid tissue (BALT) an integral structure of the lung in normal mammals, including humans? *Am. J. Respir. Cell. Mol. Biol.* 3:131.
10. Doering, D. C., J. L. Borowicz, and E. T. Crockett. 2003. Gender dimorphism in differential peripheral blood leukocyte counts in mice using cardiac, tail, foot, and saphenous vein puncture methods. *BMC Clin. Pathol.* 3:3.
11. Sitnicka, E., N. Buza-Vidas, S. Larsson, J. M. Nygren, K. Liuba, and S. E. Jacobsen. 2003. Human CD34⁺ hematopoietic stem cells capable of multilineage engrafting NOD/SCID mice express *flt3*: distinct *flt3* and *c-kit* expression and response patterns on mouse and candidate human hematopoietic stem cells. *Blood* 102:881.
12. Ganz, T. 2003. Defensins: antimicrobial peptides of innate immunity. *Nat. Rev. Immunol.* 3:710.

13. Ouellette, A. J., and M. E. Selsted. 1996. Paneth cell defensins: endogenous peptide components of intestinal host defense. *FASEB J.* 10:1280.
14. Risso, A. 2000. Leukocyte antimicrobial peptides: multifunctional effector molecules of innate immunity. *J. Leukocyte Biol.* 68:785.
15. Janeway, C. A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu. Rev. Immunol.* 20:197.
16. Bogdan, C. 2001. Nitric oxide and the immune response. *Nat. Immunol.* 2:907.
17. Weinberg, J. B. 1998. Nitric oxide production and nitric oxide synthase type 2 expression by human mononuclear phagocytes: a review. *Mol. Med.* 4:557.
18. Schneemann, M., and G. Schoedon. 2002. Species differences in macrophage NO production are important. *Nat. Immunol.* 3:102.
19. Webb, J. R., S. H. Lee, and S. M. Vidal. 2002. Genetic control of innate immune responses against cytomegalovirus: MCMV meets its match. *Genes Immun.* 3:250.
20. Lanier, L. L. 1998. NK cell receptors. *Annu. Rev. Immunol.* 16:359.
21. Monteiro, R. C., and J. G. Van De Winkel. 2003. IgA Fc receptors. *Annu. Rev. Immunol.* 21:177.
22. Daeron, M. 1997. Fc receptor biology. *Annu. Rev. Immunol.* 15:203.
23. Martin, R. M., and A. M. Lew. 1998. Is IgG2a a good Th1 marker in mice? *Immunol. Today.* 19:49.
24. Snapper, C. M., and F. D. Finkelmann. 1999. Immunoglobulin class switching. In *Fundamental Immunology*. W. E. Paul, ed. Lippincott-Raven, Philadelphia, p. 831.
25. Pappu, R., A. M. Cheng, B. Li, Q. Gong, C. Chiu, N. Griffin, M. White, B. P. Sleckman, and A. C. Chan. 1999. Requirement for B cell linker protein (BLNK) in B cell development. *Science* 286:1949.
26. Minegishi, Y., J. Rohrer, E. Coustan-Smith, H. M. Lederman, R. Pappu, D. Campana, A. C. Chan, and M. E. Conley. 1999. An essential role for BLNK in human B cell development. *Science* 286:1954.
27. Satterthwaite, A. B., and O. N. Witte. 2000. The role of Bruton's tyrosine kinase in B cell development and function: a genetic perspective. *Immunol. Rev.* 175:120.
28. Conley, M. E., J. Rohrer, L. Rapalus, E. C. Boylin, and Y. Minegishi. 2000. Defects in early B-cell development: comparing the consequences of abnormalities in pre-BCR signaling in the human and the mouse. *Immunol. Rev.* 178:75.
29. Gordon, C. J., G. Grafton, P. M. Wood, M. Larche, and R. J. Armitage. 2001. Modelling the human immune response: can mice be trusted? *Curr. Opin. Pharmacol.* 1:431.
30. Thomas, J. D., P. Sideras, C. I. Smith, I. Vorechovsky, V. Chapman, and W. E. Paul. 1993. Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. *Science* 261:355.
31. Buckley, R. H. 1999. Primary immunodeficiency diseases. In *Fundamental Immunology*. W. E. Paul, ed. Lippincott-Raven, Philadelphia, p. 1427.
32. Tokugawa, Y., M. Koyama, and J. Silver. 1997. A molecular basis for species differences in Thy-1 expression patterns. *Mol. Immunol.* 34:1263.
33. Leonard, W. J. 1999. Type I cytokines and interferons and their receptors. In *Fundamental Immunology*. W. E. Paul, ed. Lippincott-Raven, Philadelphia, p. 741.
34. Fischer, A., M. Cavazzana-Calvo, G. De Saint Basile, J. P. DeVillartay, J. P. Di Santo, C. Hivroz, F. Rieux-Laucat, and F. Le Deist. 1997. Naturally occurring primary deficiencies of the immune system. *Annu. Rev. Immunol.* 15:93.
35. Peschon, J. J., P. J. Morrissey, K. H. Grabstein, F. J. Ramsdell, E. Maraskovsky, B. C. Gliniak, L. S. Park, S. F. Ziegler, D. E. Williams, C. B. Ware, et al. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J. Exp. Med.* 180:1955.
36. Roifman, C. M., J. Zhang, D. Chitayat, and N. Sharfe. 2000. A partial deficiency of interleukin-7R α is sufficient to abrogate T-cell development and cause severe combined immunodeficiency. *Blood* 96:2803.
37. Elder, M. E., S. Skoda-Smith, T. A. Kadlecik, F. Wang, J. Wu, and A. Weiss. 2001. Distinct T cell developmental consequences in humans and mice expressing identical mutations in the DLAARN motif of ZAP-70. *J. Immunol.* 166:656.
38. Chu, D. H., N. S. van Oers, M. Malissen, J. Harris, M. Elder, and A. Weiss. 1999. Pre-T cell receptor signals are responsible for the down-regulation of Syk protein tyrosine kinase expression. *J. Immunol.* 163:2610.
39. Carding, S. R., and P. J. Egan. 2002. $\gamma\delta$ T cells: functional plasticity and heterogeneity. *Nat. Rev. Immunol.* 2:336.
40. Elbe, A., C. A. Foster, and G. Stingl. 1996. T-cell receptor $\alpha\beta$ and $\gamma\delta$ T cells in rat and human skin—are they equivalent? *Semin. Immunol.* 8:341.
41. Dutronc, Y., and S. A. Porcelli. 2002. The CD1 family and T cell recognition of lipid antigens. *Tissue Antigens.* 60:337.
42. Kaufmann, S. H. 2001. How can immunology contribute to the control of tuberculosis? *Nat. Rev. Immunol.* 1:20.
43. Flynn, J. L., and J. Chan. 2001. Immunology of tuberculosis. *Annu. Rev. Immunol.* 19:93.
44. Farrar, J. D., J. D. Smith, T. L. Murphy, S. Leung, G. R. Stark, and K. M. Murphy. 2000. Selective loss of type I interferon-induced STAT4 activation caused by a mini-satellite insertion in mouse Stat2. *Nat. Immunol.* 1:65.
45. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348.
46. Del Prete, G. F., M. De Carli, C. Mastroianni, R. Biagiotti, D. Macchia, P. Falagiani, M. Ricci, and S. Romagnani. 1991. Purified protein derivative of *Mycobacterium tuberculosis* and excretory-secretory antigen(s) of *Toxocara canis* expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. *J. Clin. Invest.* 88:346.
47. Romagnani, S. 1991. Human TH1 and TH2 subsets: doubt no more. *Immunol. Today.* 12:256.
48. Allen, J. E., and R. M. Maizels. 1997. Th1-Th2: reliable paradigm or dangerous dogma? *Immunol. Today.* 18:387.
49. Gor, D. O., N. R. Rose, and N. S. Greenspan. 2003. TH1-TH2: a procrustean paradigm. *Nat. Immunol.* 4:503.
50. Murphy, K. M. 2003. In search of the CTD. *Nat. Immunol.* 4:645.
51. Del Prete, G., M. De Carli, F. Almerigogna, M. G. Giudizi, R. Biagiotti, and S. Romagnani. 1993. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J. Immunol.* 150:353.
52. Hagan, P. 1993. IgE and protective immunity to helminth infections. *Parasite Immunol.* 15:1.
53. Pearce, E. J., and A. Sher. 1991. Functional dichotomy in the CD4⁺ T cell response to *Schistosoma mansoni*. *Exp. Parasitol.* 73:110.
54. Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol.* 14:233.
55. McAdam, A. J., R. J. Greenwald, M. A. Levin, T. Chernova, N. Malenkovich, V. Ling, G. J. Freeman, and A. H. Sharpe. 2001. ICOS is critical for CD40-mediated antibody class switching. *Nature* 409:102.
56. Tafuri, A., A. Shahinian, F. Bladt, S. K. Yoshinaga, M. Jordana, A. Wakeham, L. M. Boucher, D. Bouchard, V. S. Chan, G. Duncan, et al. 2001. ICOS is essential for effective T-helper-cell responses. *Nature* 409:105.
57. Grimbacher, B., A. Hudoff, M. Schlesier, E. Glocker, K. Warnatz, R. Drager, H. Eibel, B. Fischer, A. A. Schaffer, H. W. Mages, et al. 2003. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nat. Immunol.* 4:261.
58. Pan, J., L. Xia, and R. P. McEver. 1998. Comparison of promoters for the murine and human P-selectin genes suggests species-specific and conserved mechanisms for transcriptional regulation in endothelial cells. *J. Biol. Chem.* 273:10058.
59. Barnaba, V., C. Watts, M. de Boer, P. Lane, and A. Lanzavecchia. 1994. Professional presentation of antigen by activated human T cells. *Eur. J. Immunol.* 24:71.
60. Denton, M. D., C. S. Geehan, S. I. Alexander, M. H. Sayegh, and D. M. Briscoe. 1999. Endothelial cells modify the costimulatory capacity of transmigrating leukocytes and promote CD28-mediated CD4⁺ T cell alloactivation. *J. Exp. Med.* 190:555.
61. Taams, L. S., W. van Eden, and M. H. Wauben. 1999. Antigen presentation by T cells versus professional antigen-presenting cells (APC): differential consequences for T cell activation and subsequent T cell-APC interactions. *Eur. J. Immunol.* 29:1543.
62. Tibbitts, M. D., L. Zheng, and M. J. Lenardo. 2003. The death effector domain protein family: regulators of cellular homeostasis. *Nat. Immunol.* 4:404.
63. Chun, H. J., L. Zheng, M. Ahmad, J. Wang, C. K. Speirs, R. M. Siegel, J. K. Dale, J. Puck, J. Davis, C. G. Hall, et al. 2002. Blockade of the voltage-gated potassium channel Kv1.3 inhibits immune responses in vivo. *J. Immunol.* 158:5120.
64. Wulff, H., P. A. Calabresi, R. Allie, S. Yun, M. Pennington, C. Beeton, and K. G. Chandry. 2003. The voltage-gated Kv1.3 K⁺ channel in effector memory T cells as new target for MS. *J. Clin. Invest.* 111:1703.
65. Koo, G. C., J. T. Blake, A. Talento, M. Nguyen, S. Lin, A. Sirotna, K. Shah, K. Mulvany, D. Hora, Jr., P. Cunningham, et al. 1997. Blockade of the voltage-gated potassium channel Kv1.3 inhibits immune responses in vivo. *J. Immunol.* 158:5120.
66. Olson, T. S., and K. Ley. 2002. Chemokines and chemokine receptors in leukocyte trafficking. *Am. J. Physiol.* 283:R7.
67. Zlotnik, A., and O. Yoshie. 2000. Chemokines: a new classification system and their role in immunity. *Immunity* 12:121.
68. Pedotti, R., J. J. De Voss, L. Steinman, and S. J. Galli. 2003. Involvement of both "allergic" and "autoimmune" mechanisms in EAE, MS and other autoimmune diseases. *Trends Immunol.* 24:479.
69. Lublin, F. D., R. L. Knobler, B. Kalman, M. Goldhaber, J. Marini, M. Perrault, C. D'Imperio, J. Joseph, S. S. Alkan, and R. Korngold. 1993. Monoclonal anti- γ interferon antibodies enhance experimental allergic encephalomyelitis. *Autoimmunity* 16:267.
70. Heremans, H., C. Dillen, M. Groenen, E. Martens, and A. Billiau. 1996. Chronic relapsing experimental autoimmune encephalomyelitis (CREAE) in mice: enhancement by monoclonal antibodies against interferon- γ . *Eur. J. Immunol.* 26:2393.
71. Yednock, T. A., C. Cannon, L. C. Fritz, F. Sanchez-Madrid, L. Steinman, and N. Karin. 1992. Prevention of experimental autoimmune encephalomyelitis by antibodies against $\alpha\beta$ integrin. *Nature* 356:63.
72. Miller, D. H., O. A. Khan, W. A. Sheremata, L. D. Blumhardt, G. P. Rice, M. A. Libonati, A. J. Willmer-Hulme, C. M. Dalton, K. A. Miskiel, and P. W. O'Connor. 2003. A controlled trial of natalizumab for relapsing multiple sclerosis. *N. Engl. J. Med.* 348:15.
73. Dumonde, D. C., M. S. Pulley, F. J. Paradinas, B. M. Southcott, D. O'Connell, M. R. Robinson, F. den Hollander, and A. H. Schuur. 1982. Histological features of skin reactions to human lymphoid cell line lymphokine in patients with advanced cancer. *J. Pathol.* 138:289.
74. Crowle, A. J. 1975. Delayed hypersensitivity in the mouse. *Adv. Immunol.* 20:197.
75. Pober, J. S., M. S. Kluger, and J. S. Schenker. 2001. Human endothelial cell presentation of antigen and the homing of memory/effector T cells to skin. *Ann. NY Acad. Sci.* 941:12.
76. Murphy, L. L., M. M. Mazanet, A. C. Taylor, J. Mestas, and C. C. Hughes. 1999. Single-cell analysis of costimulation by B cells, endothelial cells, and fibroblasts demonstrates heterogeneity in responses of CD4⁺ memory T cells. *Cell Immunol.* 194:150.
77. Mestas, J., and C. C. Hughes. 2001. Endothelial cell costimulation of T cell activation through CD58-CD28 interactions involves lipid raft aggregation. *J. Immunol.* 167:4378.
78. Kreisel, D., A. S. Krupnick, K. R. Balsara, M. Riha, A. E. Gelman, S. H. Popma, W. Y. Szeto, L. A. Turka, and B. R. Rosengard. 2002. Mouse vascular endothelium activates CD8⁺ T lymphocytes in a B7-dependent fashion. *J. Immunol.* 169:6154.
79. Freitas, R. 1999. Lymphatic system. In *Nanomedicine, Volume I: Basic Capabilities*. Landes Bioscience, Georgetown, TX, p. 68.
80. Choo, J. K., J. D. Seebach, V. Nickleleit, A. Shimizu, H. Lei, D. H. Sachs, and J. C. Madsen. 1997. Species differences in the expression of major histocompatibility

- complex class II antigens on coronary artery endothelium: implications for cell-mediated xenoreactivity. *Transplantation* 64:1315.
81. Hughes, C. C., C. O. Savage, and J. S. Pober. 1990. Endothelial cells augment T cell interleukin 2 production by a contact-dependent mechanism involving CD2/LFA-3 interaction. *J. Exp. Med.* 171:1453.
 82. van der Merwe, P. A. 1999. A subtle role for CD2 in T cell antigen recognition. *J. Exp. Med.* 190:1371.
 83. Karmann, K., C. C. Hughes, J. Schechner, W. C. Fanslow, and J. S. Pober. 1995. CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. *Proc. Natl. Acad. Sci. USA* 92:4342.
 84. Ensminger, S. M., B. M. Spriewald, T. Fischlein, M. Weyand, P. J. Morris, and K. J. Wood. 2003. Mouse endothelial CD40 expression does not play a role during the development of transplant arteriosclerosis. *Endothelium* 10:111.
 85. McDade, T. W., and C. M. Worthman. 1999. Evolutionary process and the ecology of human immune function. *Am. J. Human Biol.* 11:705.
 86. Martin, A. M., J. K. Kulski, C. Witt, P. Pontarotti, and F. T. Christiansen. 2002. Leukocyte Ig-like receptor complex (LRC) in mice and men. *Trends Immunol.* 23:81.
 87. Gurtner, G. C., V. Davis, H. Li, M. J. McCoy, A. Sharpe, and M. I. Cybulsky. 1995. Targeted disruption of the murine VCAM1 gene: essential role of VCAM-1 in chorioallantoic fusion and placentation. *Genes Dev.* 9:1.
 88. Rehli, M. 2002. Of mice and men: species variations of Toll-like receptor expression. *Trends Immunol.* 23:375.
 89. Heinz, S., V. Haehnel, M. Karaghiosoff, L. Schwarzfischer, M. Muller, S. W. Krause, and M. Rehli. 2003. Species-specific regulation of Toll-like receptor 3 genes in men and mice. *J. Biol. Chem.* 278:21502.
 90. Lund, J., A. Sato, S. Akira, R. Medzhitov, and A. Iwasaki. 2003. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *J. Exp. Med.* 198:513.
 91. Trevani, A. S., A. Chorny, G. Salamone, M. Vermeulen, R. Gamberale, J. Schettini, S. Raiden, and J. Geffner. 2003. Bacterial DNA activates human neutrophils by a CpG-independent pathway. *Eur. J. Immunol.* 33:3164.
 92. Varki, A. 2001. Loss of *N*-glycolylneuraminic acid in humans: mechanisms, consequences, and implications for hominid evolution. *Am. J. Phys. Anthropol.* 33(Suppl.):54.
 93. Brinkman-Van der Linden, E. C., T. Angata, S. A. Reynolds, L. D. Powell, S. M. Hedrick, and A. Varki. 2003. CD33/Siglec-3 binding specificity, expression pattern, and consequences of gene deletion in mice. *Mol. Cell Biol.* 23:4199.
 94. Cunliffe, R. N., F. R. Rose, J. Keyte, L. Abberley, W. C. Chan, and Y. R. Mahida. 2001. Human defensin 5 is stored in precursor form in normal Paneth cells and is expressed by some villous epithelial cells and by metaplastic Paneth cells in the colon in inflammatory bowel disease. *Gut* 48:176.
 95. Ghosh, D., E. Porter, B. Shen, S. K. Lee, D. Wilk, J. Drazba, S. P. Yadav, J. W. Crabb, T. Ganz, and C. L. Bevins. 2002. Paneth cell trypsin is the processing enzyme for human defensin-5. *Nat. Immunol.* 3:583.
 96. Crocker, P. R., W. A. Jefferies, S. J. Clark, L. P. Chung, and S. Gordon. 1987. Species heterogeneity in macrophage expression of the CD4 antigen. *J. Exp. Med.* 166:613.
 97. Kaufmann, S. H. 1996. γ/δ and other unconventional T lymphocytes: what do they see and what do they do? *Proc. Natl. Acad. Sci. USA* 93:2272.
 98. Raulet, D. H. 2003. Roles of the NKG2D immunoreceptor and its ligands. *Nat. Rev. Immunol.* 3:781.
 99. Gao, J. L., and P. M. Murphy. 1993. Species and subtype variants of the *N*-formyl peptide chemotactic receptor reveal multiple important functional domains. *J. Biol. Chem.* 268:25395.
 100. Zemlin, M., M. Klinger, J. Link, C. Zemlin, K. Bauer, J. A. Engler, H. W. Schroeder, Jr., and P. M. Kirkham. 2003. Expressed murine and human CDR-H3 intervals of equal length exhibit distinct repertoires that differ in their amino acid composition and predicted range of structures. *J. Mol. Biol.* 334:733.
 101. Chapoval, A. I., J. Ni, J. S. Lau, R. A. Wilcox, D. B. Flies, D. Liu, H. Dong, G. L. Sica, G. Zhu, K. Tamada, and L. Chen. 2001. B7-H3: a costimulatory molecule for T cell activation and IFN- γ production. *Nat. Immunol.* 2:269.
 102. Suh, W. K., B. U. Gajewska, H. Okada, M. A. Gronski, E. M. Bertram, W. Dawicki, G. S. Duncan, J. Bukczynski, S. Plyte, A. Elia, et al. 2003. The B7 family member
 103. Geijtenbeek, T. B., R. Torensma, S. J. van Vliet, G. C. van Duinjhoven, G. J. Adema, Y. van Kooyk, and C. G. Figdor. 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* 100:575.
 104. van Kooyk, Y., and T. B. Geijtenbeek. 2002. A novel adhesion pathway that regulates dendritic cell trafficking and T cell interactions. *Immunol. Rev.* 186:47.
 105. Crommie, D., and S. D. Rosen. 1995. Biosynthesis of GlyCAM-1, a mucin-like ligand for L-selectin. *J. Biol. Chem.* 270:22614.
 106. Correa, I., T. Plunkett, A. Vlad, A. Mungul, J. Candelora-Kettel, J. M. Burchell, J. Taylor-Papadimitriou, and O. J. Finn. 2003. Form and pattern of MUC1 expression on T cells activated in vivo or in vitro suggests a function in T-cell migration. *Immunology* 108:32.



IL-10 fails to abrogate experimental autoimmune encephalomyelitis.

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Mice adoptively-sensitized to develop chronic relapsing experimental autoimmune encephalomyelitis (EAE), a model for the human demyelinating condition, multiple sclerosis (MS), were given injections of recombinant human IL-10 at various timepoints post-sensitization in an attempt to abrogate disease development. IL-10 is a Th2 immunomodulatory cytokine with known down-regulatory effects upon Th1 responses and macrophages. Contrary to a previous report on EAE and the predicted outcome, after repeated experiments, IL-10 was found to elicit a worsening or no effect upon EAE in the mouse. Animals were studied clinically, histopathologically and immunocytochemically. On no occasion was disease ameliorated by IL-10. Pretreatment with IL-10 of lymph node cells used to transfer EAE had no effect upon disease outcome, indicating that the cells were already committed effectors. Administration of anti-IL-10 monoclonal antibody before onset of signs had no effect when given early post-sensitization and caused marked worsening when given immediately before onset of signs. In the context of this autoimmune demyelinating model, these results suggest that IL-10 alone is insufficient to reverse the effector response and indeed may serve to enhance the cascade of events in EAE.

Transgenic Interleukin 10 Prevents Induction of Experimental Autoimmune Encephalomyelitis

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Summary

The effectiveness of interleukin 10 (IL-10) in the treatment of autoimmune-mediated central nervous system inflammation is controversial. Studies of the model system, experimental autoimmune encephalomyelitis (EAE), using various routes, regimens, and delivery methods of IL-10 suggest that these variables may affect its immunoregulatory function. To study the influence of these factors on IL-10 regulation of EAE pathogenesis, we have analyzed transgenic mice expressing human IL-10 (hIL-10) transgene under the control of a class II major histocompatibility complex (MHC) promoter. The hIL-10 transgenic mice are highly resistant to EAE induced by active immunization, and this resistance appears to be mediated by suppression of autoreactive T cell function. Myelin-reactive T helper 1 cells are induced but non-pathogenic in the IL-10 transgenic mice. Antibody depletion confirmed that EAE resistance is dependent on the presence of the transgenic IL-10. Mice expressing the hIL-10 transgene but not the endogenous murine IL-10 gene demonstrated that transgenic IL-10 from MHC class II-expressing cells is sufficient to block induction of EAE. This study demonstrates that IL-10 can prevent EAE completely if present at appropriate levels and times during disease induction.

Key words: major histocompatibility complex class II promoter-regulated interleukin 10 • myelin-reactive T cells • immune regulation • autoimmunity

IL-10 is an immune regulatory factor that has potential therapeutic value for organ-specific autoimmune diseases (1). Administration of rIL-10 reduces disease in experimental models of diabetes, rheumatoid arthritis, and inflammatory bowel disease (2–6); however, its utility in autoimmune encephalomyelitis is unclear. Studies using various routes, regimens, and delivery methods of rIL-10 have yielded conflicting results. In an adoptive transfer model of experimental autoimmune encephalomyelitis (EAE), intravenous injection of rIL-10 exacerbated rather than suppressed disease (7). In contrast, subcutaneous rIL-10 treatment partially inhibited disease in rat and mouse models of EAE induced by active immunization (8, 9). In these active immunization models, daily rIL-10 treatment regimens from the day of immunization to just before the expected day of disease onset were necessary for significant EAE inhibition. These results suggest that systemic rIL-10 treatment may inhibit EAE either by suppressing *in vivo* development or migration of encephalitogenic Th1 cells or by

inhibiting the inflammatory effector function of these Th1 cells. Recent studies of targeted delivery of IL-10 to the central nervous system (CNS) have also yielded conflicting results. Intracranial injection of soluble rIL-10 or plasmids containing a retroviral promoter-directed IL-10 cDNA at day 12 after active immunization did not suppress EAE (10). Adoptive transfer of retroviral transduced myelin basic protein (MBP)-specific T cell hybridoma also did not inhibit EAE (11). In contrast, antigen-inducible IL-10 produced by proteolipid protein (PLP)-specific T memory cells suppressed EAE when adoptively transferred to PLP peptide-immunized mice 1 d before expected disease onset (12). These results suggest that the timing of IL-10 production and possibly the localization of IL-10 may critically affect its immunoregulatory function.

To study the influence of these factors on IL-10 regulation of EAE pathogenesis, we have analyzed transgenic mice expressing human IL-10 (hIL-10) under the control of a class II MHC promoter. hIL-10 is fully active in mice

(1), and the species difference allows for the specific detection and depletion of transgenic and endogenous IL-10. In the present study, we have demonstrated that hIL-10 transgenic (hIL-10Tg) mice are resistant to EAE induced by active immunization and that this resistance is a consequence of a reduced autoreactive Th1 response.

Materials and Methods

Mice. SJL/J and CSJLF1/J mice were obtained from The Jackson Laboratory. BALB/cAnN mice were obtained from Taconic Farms, Inc. hIL-10Tg mice were constructed using a hIL-10 cDNA sequence regulated by a class II MHC E α promoter sequence (13). For this study, male hemizygous BALB/c IL-10Tg mice were backcrossed with female BALB/cAnN mice to generate hIL-10Tg and nonTg littermates. SJL \times BALB/c F1 transgene-positive and nonTg littermates were generated by crossing male hemizygous hIL-10Tg BALB/cAnN mice with female SJL/J mice. To generate mice expressing the hIL-10 transgene but lacking endogenous murine IL-10, the hIL-10 transgene was backcrossed onto the BALB/cAnN IL-10KO background. Male BALB/cAnN hemizygous hIL-10Tg IL-10KO mice were bred with female BALB/cAnN IL-10KO mice to produce transgene-positive and nonTg littermate mice on the IL-10KO background.

Induction of EAE. Mouse spinal cord homogenate (MSCH) was prepared from 8–12-wk-old BALB/cAnN mice as previously described (14). Bovine MBP was obtained from Sigma Chemical Co. For active induction of EAE, mice were immunized intradermally with 2.5 mg of MSCH and 200 μ g of *Mycobacterium tuberculosis* (strain H37RA; Difco) at days 0 and 7 as described (14). Mice were examined and scored for clinical signs of EAE, and routine histopathological analyses of hematoxylin and eosin- or Luxol fast blue-stained paraffin sections were performed in a masked fashion as described (14).

Antibodies. All mAb were purified by column chromatography from either ascites fluid or tissue culture supernatants and contained <4 EU endotoxin/mg protein. 1B1.2 is a blocking mAb reactive with mouse IL-10 receptor used in *in vitro* cultures (15). The following mAbs were used *in vivo*: JES3-9D7 (rat IgG1), a hIL-10-specific mAb that does not cross-react with mouse IL-10 (16); and GL113 (rat IgG1), an isotype control mAb reactive with β -galactosidase. For *in vivo* mAb treatment, mice were injected i.p. with 1 mg mAb/dose in 100 μ l of PBS. Each animal received three injections, once per week.

Cell Purification. CD4 $^{+}$ draining lymph node (DLN) cells were purified by positive selection using MACS $^{\circ}$ L3T4 micro-

beads and MiniMACS $^{\circ}$ columns (Miltenyi Biotec). The microbead-labeled cell suspensions were processed through the magnetic column twice, and the purity was routinely >95% CD4 $^{+}$ cells. To prepare T cell-depleted APC, spleen cells from naive CSJLF1/J mice were depleted of CD4 (GK1.5, 20 μ g/ml)- and CD8 (2-43, 20 μ g/ml)-staining cells by negative selection using anti-rat Ig-coated Dynabeads (Dyna).

Cell Culture and Cytokine Detection. Mice were immunized with MSCH following the same procedure as for active induction of EAE. 10 d after immunization, DLN cells (4×10^6 /ml) and purified CD4 $^{+}$ DLN cells (10^6 /ml) were stimulated with 50 μ g/ml of MBP as described (14). Anti-IL-10 receptor mAb (1B1.2, 10 μ g/ml) was added to cultures where indicated. Culture supernatants were harvested after 60 h and levels of IFN- γ and IL-4 were determined using a sandwich ELISA technique as described (14).

Results

Transgenic IL-10 Prevents Induction of EAE. For the initial study, hIL-10Tg mice and nonTg littermate controls on the BALB/cAnN genetic background were compared for EAE susceptibility. Although BALB/c mice are generally considered to be EAE resistant, it has been reported that BALB/cAnN and BALB/cByJ substrains are susceptible to actively induced EAE (17). Consistent with these results, EAE was reproducibly induced in these strains of mice by intradermal immunization on days 0 and 7 with MSCH in CFA (84%, $n = 86$; average EAE grade = 3.2; day of disease onset = 17–30 d after immunization). With this immunization, hIL-10Tg mice were completely EAE resistant (0/23 mice), whereas control littermate mice were highly EAE susceptible and exhibited the same EAE severity as wild-type BALB/cAnN and SJL/J mice (Fig. 1, A and B). No inflammatory infiltrates were found in the spinal cords of EAE-resistant Tg mice, whereas intense inflammatory infiltrates and demyelination were found in the white matter of EAE-susceptible nonTg littermate mice (Fig. 2).

To confirm these findings in a more conventional model of EAE, hemizygous male BALB/c hIL-10Tg mice were crossed with SJL/J mice to generate transgene-positive and nonTg littermates. These F1 mice were compared to CSJLF1/J, which have a highly predictable disease onset at 14 d after immunization with MSCH and a relatively strong *in vitro* recall response to MBP. Transgene-positive

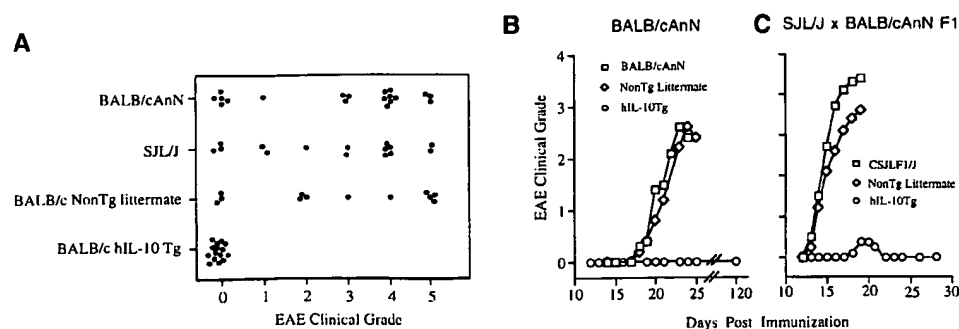


Figure 1. hIL-10Tg mice are EAE resistant. (A) Circles represent peak severity of clinical disease for individual mice on the indicated genetic backgrounds. Data compiled from three separate experiments. (B) Average severity score for hIL-10Tg mice on the BALB/cAnN background. One representative experiment of five is shown. (C) Average severity score for hIL-10Tg mice on SJL/J \times BALB/cAnN F1 background. One representative experiment of four is shown.

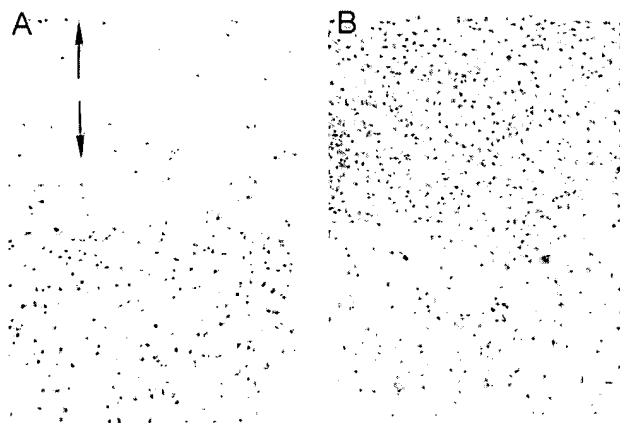


Figure 2. Histology of spinal cords of hIL-10Tg mice and nonTg littermates immunized to induce EAE. (A) hIL-10Tg mice were EAE resistant and spinal cords showed no inflammatory infiltrates or plaques of demyelination in the white matter (normal white matter outlined by arrows). (B) NonTg littermates were highly EAE susceptible and showed inflammatory infiltrates and extensive demyelination in the white matter. Luxol fast blue stain with hematoxylin counterstain. $\times 125$.

SJL/J \times BALB/cAnN F1 mice had a low EAE incidence (4/55 mice) and disease severity compared to nonTg littermate control mice (43/46 mice), which were highly susceptible to EAE. The nonTg littermates exhibited an EAE severity and disease course similar to wild-type CSJLF1/J mice (Fig. 1 C). No difference in EAE severity or day of disease onset was observed when comparing CSJLF1/J with SJL/J mice (data not shown).

Transgenic IL-10 Must Be Present during the Induction of Disease to Prevent EAE. To show that disease resistance was due to the direct effects of the transgenic IL-10, immunized transgenic SJL/J \times BALB/cAnN F1 mice were injected with either JES3-9D7 mAb (hIL-10-specific mAb [16] that does not cross-react with mouse IL-10) or GL113 mAb (an isotype-matched mAb control). Tg mice receiving the first anti-hIL-10 mAb injection on the day of immunization were susceptible to EAE, whereas the isotype control mAb-injected mice were resistant to EAE (Fig. 3 A). This result suggests that EAE resistance of hIL-10Tg mice was the consequence of hIL-10 present during immunization, rather than of developmental effects of the transgene. A consistent delay in the day of disease onset was found in the anti-hIL-10 mAb-treated mice compared to

control littermates (Fig. 3 A). To determine the cause of this delay, anti-hIL-10 mAb treatment was initiated 8 d before MSCH immunization. With this treatment regimen, the EAE clinical grade and day of disease onset for hIL-10Tg and control littermate mice were indistinguishable (Fig. 3 B). Similar results were obtained when hIL-10Tg mice on the BALB/cAnN genetic background were treated with the anti-hIL-10 mAb (data not shown).

MBP-specific Th1 Cells Were Generated Normally in IL-10Tg Mice. Three possible mechanisms for the regulatory effect of IL-10Tg in EAE are as follows: inhibition of the initial development of autoreactive Th1 cells, active inhibition of Th1 cells by IL-10, or immune deviation toward a Th2-type response. To test these, the cytokine secretion profiles of DLN cells from MSCH-immunized SJL \times BALB/c F1 hIL-10Tg and control littermate mice were analyzed. 10 d after immunization, DLN cells were prepared for culture in the presence of MBP or MBP plus anti-IL-10 receptor mAb. After 3 d, the culture supernatants were tested for secreted IFN- γ and IL-4. Cells from nonTg littermate mice stimulated with MBP secreted higher levels of IFN- γ than cells from Tg mice, a result consistent with the association of EAE pathogenesis with IFN- γ production (Fig. 4). However, when stimulated with MBP in the presence of anti-IL-10 receptor mAb, cells from both hIL-10Tg mice and littermate controls produced similar levels of IFN- γ . To eliminate APC differences between hIL-10Tg and control littermate mice, CD4 $^{+}$ T cells were purified and cultured with irradiated, T-depleted splenocytes from CSJLF1/J donor mice. In the absence of APC-derived hIL-10Tg, T cells from both hIL-10Tg and control littermate mice produced equivalent levels of IFN- γ (Fig. 4 B). No MBP-specific IL-4 production was detected in cultures of DLN cells or CD4 $^{+}$ T cells from hIL-10Tg or control littermate mice, suggesting that EAE resistance in the Tg mice was not associated with a Th2-type response (data not shown). These results support the interpretation that myelin-reactive Th1 cells were induced in the hIL-10Tg mice and suggest that their ability to exert an effector function was actively suppressed by IL-10 in vivo.

hIL-10Tg in the Absence of Endogenous Mouse IL-10 Can Prevent EAE. To assess whether T cell-derived endogenous mouse IL-10 was required for EAE resistance, the IL-10Tg mice were backcrossed onto an IL-10 null (IL-10KO) background to generate mice that expressed hIL-10Tg but

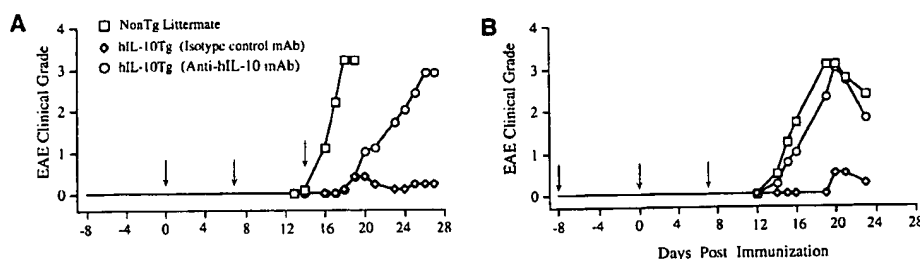


Figure 3. Induction of EAE in hIL-10Tg mice treated with anti-hIL-10 mAb. Days of mAb treatment are indicated by arrows. (A) Tg mice were injected with anti-hIL-10 mAb (1 mg/dose) or isotype-matched mAb starting at the day of MSCH immunization. One representative experiment out of five is shown. (B) Mice were injected with the mAb starting 8 d before MSCH immunization. One representative experiment out of three is shown.

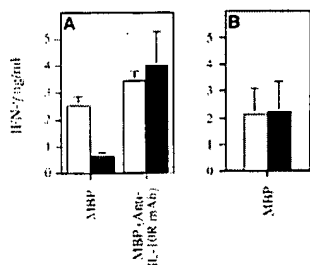


Figure 4. MBP-specific Th1 cells are induced in hIL-10Tg mice. hIL-10Tg mice (black bar) and control littermate mice (gray bar) were immunized twice at 1-wk intervals with MSCH emulsified in CFA. (A) 3 d after the last immunization, DLN cells from hIL-10Tg or control littermate mice were stimulated with MBP or MBP plus anti-IL-10 receptor mAb. One representative

experiment out of three is shown. (B) CD4⁺ T cells were purified and stimulated in vitro with MBP plus irradiated T cell-depleted splenocytes from control CSJLF1/J mice for 60 h. The amount of IFN-γ in the supernatants was measured by ELISA. One representative experiment out of two is shown. The results are the mean of three or four individual mice/group ± SEM.

lacked endogenous mouse IL-10. Because murine T cells do not express MHC class II molecules, no T cell-derived IL-10 is present in these mice. The hIL-10Tg IL-10KO mice and nonTg IL-10KO littermates were tested for susceptibility to EAE. Transgene-positive IL-10KO mice, which only have MHC class II-positive cell-derived IL-10, were completely resistant to EAE, whereas nonTg IL-10KO littermates were highly susceptible to EAE (Fig. 5). No difference in EAE susceptibility was observed when nonTg IL-10KO littermates were compared with BALB/cAnN IL-10KO mice (data not shown). Treatment with anti-hIL-10 mAb reversed this protection in the Tg IL-10KO mice, which became as susceptible to EAE as nonTg IL-10KO littermates (Fig. 5). To determine the extent of the protection provided by the IL-10Tg in the absence of endogenous murine IL-10, the CNS of the three groups of mice from Fig. 5 were examined. Intense inflammatory infiltrates and extensive demyelination as determined by the loss of Luxol fast blue staining were found in the white matter of nonTg IL-10KO littermates, consistent with the

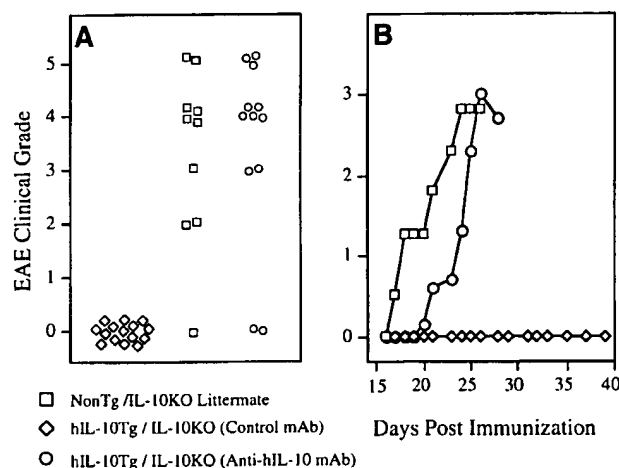


Figure 5. hIL-10Tg mice on the IL-10KO genetic background are EAE resistant. (A) Peak severity scores of individual mice treated with isotype control mAb or anti-hIL-10 mAb (1 mg/dose) on days 0, 7, and 14. Results shown are compiled from two separate experiments. (B) Average severity score over time for the three groups of mice shown in A.

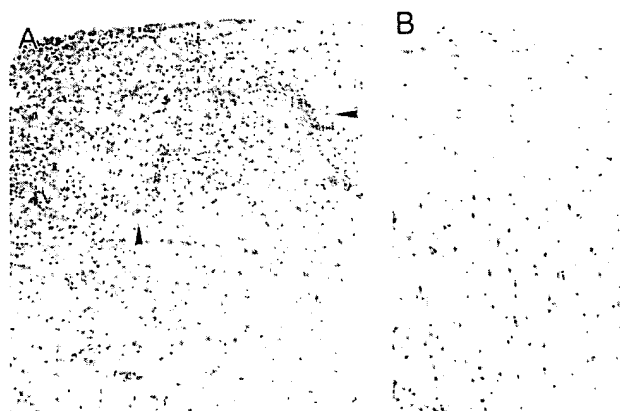


Figure 6. Histology of spinal cords of hIL-10Tg mice on the IL-10KO background shown in Fig. 5. (A) NonTg IL-10KO mice were EAE susceptible and showed inflammatory infiltrates and plaques of demyelination in spinal cords (arrowheads). (B) hIL-10Tg IL-10KO mice were EAE resistant and their spinal cords showed no inflammatory infiltrates or demyelination. Luxol fast blue stain with hematoxylin counterstain. ×125.

clinical disease (Fig. 6 A). In contrast, the CNS of the EAE-resistant mice expressing the IL-10 transgene but lacking endogenous murine IL-10 were completely free of inflammatory infiltrates and demyelination (Fig. 6 B). Examination of the CNS of hIL-10 transgene-positive IL-10KO mice treated with anti-hIL-10 mAb showed extensive inflammation and demyelination similar to the pathological changes found in the nonTg littermates (data not shown). These data suggest that APC production of IL-10 alone, despite the complete absence of T cell-produced IL-10, is sufficient to protect mice from EAE.

Discussion

In this study, we have demonstrated that Tg mice expressing hIL-10 under the control of the MHC class II promoter are completely resistant to induction of EAE and that this resistance is independent of T cell-derived IL-10. The use of human IL-10, which has similar specific activity to murine IL-10 (1, 2), allowed for the specific measurement and inhibition of Tg and endogenous IL-10. Treatment of MSCH-immunized mice with neutralizing anti-hIL-10 at the time of immunization rendered transgene-positive mice fully susceptible to EAE, although the disease onset was delayed by several days (Figs. 3 and 5). Treatment of hIL-10Tg mice with anti-hIL-10 mAb 8 d before MSCH immunization resulted in kinetics of disease onset identical to that of nonTg littermates, suggesting that the elevated IL-10 level before immunization can also influence the response to antigen, consistent with our previous study showing that IL-10 has significant effects on the function of APC prior to antigen-induced activation (18). Furthermore, APC-derived IL-10Tg is sufficient for EAE prevention, as shown by the resistance of transgene-positive BALB/c mice homozygous for a mutated mouse IL-10 gene (Fig. 5). Therefore, IL-10 need not be provided by T cells to prevent EAE.

Several observations suggest that myelin-reactive Th1 cells are induced in hIL-10Tg mice despite the absence of disease. Similar levels of IFN- γ were produced by purified, MBP-specific CD4 $^{+}$ cells from EAE-resistant hIL-10Tg mice and EAE-susceptible nonTg littermates when stimulated in vitro with wild-type APC and MBP (Fig. 4). No MBP-induced IL-4 was produced by T cells from Tg or nonTg mice, suggesting that EAE resistance was not associated with a Th2 response. These results are consistent with a report showing that, although systemic treatment with rIL-10 decreased the severity of EAE in SJL mice, PLP-specific Th1 cells were induced and no PLP-specific Th2 cells were found (9). These data are also in agreement with the original finding that IL-10 does not substantially inhibit the development of Th1 cells but does inhibit their effector function (1, 19).

EAE in BALB/c and SJL \times BALB/c F1 mice is characterized by an intense inflammatory infiltrate found predominantly in the white matter of the CNS and occasionally extending into the gray matter region, causing neuronal destruction and death (not shown). The absence of an inflammatory infiltrate in the CNS of EAE-resistant Tg mice (Figs. 2 and 6) suggests that either autoreactive T cells did not enter the CNS or the few T cells entering the CNS were not able to recruit additional inflammatory cells. In vitro, IL-10 has been shown to inhibit antigen presentation and production of IL-1, IL-6, TNF- α , CD80, and CD86 by LPS- and/or IFN- γ -activated microglia (20, 21). Therefore, it is possible that IL-10Tg in the CNS may inhibit microglia antigen presentation and activation of myelin-reactive Th1 cells.

Several parameters, including the amount of IL-10 and the mode of gene regulation in specific local regions, may

account for the effectiveness of IL-10Tg in inhibition of EAE. hIL-10 was detectable in the serum of the hIL-10Tg mice at levels of 400–700 pg/ml (13). In vitro, hIL-10 and mouse IL-10 were detected in the lymph node cell cultures at similar levels of 2–6 ng/ml (data not shown). Thus, IL-10Tg was not present systemically at high levels even though it significantly protected mice from EAE.

Two separate studies have shown that IL-10 regulated by lymphocyte-specific promoters can inhibit EAE. Adoptive transfer of PLP-specific memory T cells expressing IL-2 promoter-regulated IL-10 partially reduced or reversed disease in PLP-immunized recipient mice (12). FVB \times SJL F1 mice expressing Tg murine IL-10 under the lymphocyte-specific CD2 promoter are resistant to EAE induced by PLP immunization (22). These results and the findings in the present study suggest that, in addition to lymphocyte-derived IL-10, MHC class II-positive cell-derived IL-10Tg, which may be upregulated early in an inflammatory response, can dramatically protect mice from EAE. Furthermore, our preliminary results show that adoptive transfer of MBP-specific encephalitogenic Th1 cells can not induce EAE in hIL-10Tg mice, suggesting a role for local inhibition of Th1 cells by MHC class II promoter-regulated IL-10Tg within the CNS (data not shown). These studies suggest that IL-10, applied with appropriate localization, in appropriate amounts, and at the appropriate time, can completely protect animals from EAE, despite their generation of potentially pathogenic Th1-like cells. This model should permit a more detailed understanding of the conditions necessary for IL-10 inhibition of autoimmune-mediated CNS inflammation, as this insight will provide information about how to use IL-10 in therapeutic situations.

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References

- Moore, K.W., A. O'Garra, R. de Waal Malefyt, P. Vieira, and T.R. Mosmann. 1993. Interleukin-10. *Annu. Rev. Immunol.* 11:165–190.
- Pennline, K.J., E. Roque-Gaffney, and M. Monahan. 1994. Recombinant human IL-10 prevents the onset of diabetes in the nonobese diabetic mouse. *Clin. Immunol. Immunopathol.* 71:169–175.
- Balasa, B., and N. Sarvetnick. 1996. The paradoxical effects of interleukin 10 in the immunoregulation of autoimmune diabetes. *J. Autoimmun.* 9:283–286.
- Tanaka, Y., T. Otsuka, T. Hotokebuchi, H. Miyahara, H. Nakashima, S. Kuga, Y. Nemoto, H. Niino, and Y. Niho. 1996. Effect of IL-10 on collagen-induced arthritis in mice. *Inflamm. Res.* 45:283–288.
- Persson, S., A. Mikulowska, S. Narula, A. O'Garra, and R. Holmdahl. 1996. Interleukin-10 suppresses the development of collagen type II-induced arthritis and ameliorates sustained arthritis in rats. *Scand. J. Immunol.* 44:607–614.
- Powrie, F., M.W. Leach, S. Mauze, S. Menon, L.B. Caddle, and R.L. Coffman. 1994. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RB hi CD4 $^{+}$ T cells. *Immunity* 1:553–562.

7. Cannella, B., Y.L. Gao, C. Brosnan, and C.S. Raine. 1996. IL-10 fails to abrogate experimental autoimmune encephalomyelitis. *J. Neurosci. Res.* 45:735-746.
8. Rott, O., B. Fleischer, and E. Cash. 1994. Interleukin-10 prevents experimental allergic encephalomyelitis in rats. *Eur. J. Immunol.* 24:1434-1440.
9. Nagelkerken, L., B. Blauw, and M. Tielemans. 1997. IL-4 abrogates the inhibitory effect of IL-10 on the development of experimental allergic encephalomyelitis in SJL mice. *Int. Immunol.* 9:1243-1251.
10. Croxford, J.L., K. Triantaphyllopoulos, O.L. Podhajcer, M. Feldmann, D. Baker, and Y. Chernajovsky. 1998. Cytokine gene therapy in experimental allergic encephalomyelitis by injection of plasmid DNA-cationic liposome complex into the central nervous system. *J. Immunol.* 160:5181-5187.
11. Shaw, M.K., J.B. Lorens, A. Dhawan, R. DaiCanto, H.Y. Tse, A.B. Tran, C. Bonpane, S.L. Eswaran, S. Brocke, N. Sarvetnick, et al. 1997. Local delivery of interleukin 4 by retrovirus-transduced T lymphocytes ameliorates experimental autoimmune encephalomyelitis. *J. Exp. Med.* 185:1711-1714.
12. Mathisen, P.M., M. Yu, J.M. Johnson, J.A. Drazba, and V.K. Tuohy. 1997. Treatment of experimental autoimmune encephalomyelitis with genetically modified memory T cells. *J. Exp. Med.* 186:159-164.
13. Groux, H., F. Cottrez, M. Rouleau, S. Mauze, S. Antonenko, S. Hurst, T. McNeil, M. Bigler, M.-G. Roncarolo, and R.L. Coffman. 1999. A transgenic model to analyze the immunoregulatory role of IL-10 secreted by antigen-presenting cells. *J. Immunol.* 162:1723-1729.
14. Cua, D.J., D.R. Hinton, and S.A. Stohlman. 1995. Self-antigen-induced Th2 responses in experimental allergic encephalomyelitis (EAE)-resistant mice. Th2-mediated suppression of autoimmune disease. *J. Immunol.* 155:4052-4059.
15. O'Farrell, A.M., Y. Liu, K.W. Moore, and A.L. Muir. 1998. IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways. *EMBO J.* 17:1006-1018.
16. Abrams, J.S., M.G. Roncarolo, H. Yssel, U. Andersson, G.J. Gleich, and J.E. Silver. 1992. Strategies of anti-cytokine monoclonal antibody development: immunoassay of IL-10 and IL-5 in clinical samples. *Immunol. Rev.* 127:5-24.
17. Teuscher, C., E.P. Blankenhorn, and W.F. Hickey. 1987. Differential susceptibility to actively induced experimental allergic encephalomyelitis and experimental allergic orchitis among BALB/c substrains. *Cell Immunol.* 110:294-304.
18. Cua, D.J., R.L. Coffman, and S.A. Stohlman. 1996. Exposure to T helper 2 cytokines in vivo before encounter with antigen selects for T helper subsets via alterations in antigen-presenting cell function. *J. Immunol.* 157:2830-2836.
19. Fiorentino, D.F., A. Zlotnik, P. Vieira, T.R. Mosmann, M. Howard, K.W. Moore, and A. O'Garra. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J. Immunol.* 146:3444-3451.
20. Frei, K., H. Lins, C. Schwerdel, and A. Fontana. 1994. Antigen presentation in the central nervous system. The inhibitory effect of IL-10 on MHC class II expression and production of cytokines depends on the inducing signals and the type of cell analyzed. *J. Immunol.* 152:2720-2728.
21. Menendez Iglesias, B., J. Cerase, C. Ceracchini, G. Levi, and F. Aloisi. 1997. Analysis of B7-1 and B7-2 costimulatory ligands in cultured mouse microglia: upregulation by interferon-gamma and lipopolysaccharide and downregulation by interleukin-10, prostaglandin E2 and cyclic AMP-elevating agents. *J. Neuroimmunol.* 72:83-93.
22. Bettelli, E., M.P. Das, E.D. Howard, H.L. Weiner, R.A. Sobel, and V.K. Kuchroo. 1998. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J. Immunol.* 161:3299-3306.

Myelin specific Th1 cells are necessary for post-traumatic protective autoimmunity

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Abstract

Myelin-specific encephalitogenic T cells, when passively transferred into rats or mice, cause an experimental autoimmune disease. Previous studies by our group have shown that (a) the same cells also significantly reduce post-traumatic degeneration in these animals after injury to the central nervous system, (b) this beneficial autoimmunity is a physiological response, and (c) animals differ in their ability to resist injurious conditions, and the ability to resist post-traumatic degeneration correlates with resistance to the development of an autoimmune disease. Here we show that optic nerve neurons in both resistant and susceptible rat strains can be protected from secondary degeneration after crush injury by immunization with myelin basic protein emulsified in complete or incomplete Freund's adjuvant. We provide evidence that potentially destructive autoimmunity (causing autoimmune disease) and beneficial autoimmunity (causing improved neuronal survival) both result from activity of the same myelin-specific, proinflammatory Th1 cells. We further show that following passive transfer of such Th1 cells, the expression of their beneficial potential depends on the activity of an additional T cell (CD4⁺) population. By identifying the additional cellular component of autoimmune neuroprotection, we may be able to take meaningful steps toward achieving neuroprotection without risk of accompanying autoimmune disease.

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Keywords: Myelin specific Th1 cell; Post-traumatic protective autoimmunity; Neuroprotection

1. Introduction

Autoimmunity has long been viewed as a destructive response to harmful self-components. However, our research group recently provided evidence that autoimmunity is the body's endogenous response to a central nervous system (CNS) injury, and that its purpose is beneficial (Schwartz and Kipnis, 2001; Yoles et al., 2001). This conclusion is based on experimental findings in rodents, demonstrating that (a) passive transfer of encephalitogenic (disease-inducing) T cells reactive to myelin basic protein (MBP) reduces post-injury neuronal losses relative to those of controls (Butovsky et al., 2001; Hauben et al., 2000a,b; Moalem et al., 1999, 2000b); (b) immune neuroprotection can also be exerted by transfer of autoimmune T cells that recognize non-encephalitogenic epitopes (Kipnis et al.,

2000; Moalem et al., 1999); (c) the beneficial effect of these autoreactive T cells is not merely the result of an experimental manipulation, but is a physiological response to CNS insult (Schwartz and Kipnis, 2001; Yoles et al., 2001); and (d) the ability to spontaneously exhibit this endogenous autoimmune neuroprotection varies among individuals and is directly correlated to the individual's genetically determined resistance to the development of the transient monophasic autoimmune disease known as experimental autoimmune encephalomyelitis (EAE) (Kipnis et al., 2001; Lundberg et al., 2001; Schwartz and Kipnis, 2001).

In view of these findings, we were interested in determining the nature of the relationship between encephalitogenic and beneficial autoimmune T cells. We also wanted to find out whether the two functions (disease induction and neuroprotection) are served by the same cells, and if so, what makes them encephalitogenic in certain circumstances and beneficial in others (Cohen and Schwartz, 1999; Hafler and Weiner, 1987; Hohlfeld et al., 2000; Steinman, 2001; Wekerle, 1998).

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In this study, we show that induction of an autoimmune disease is not a necessary condition for autoimmune neuroprotection. Thus, for example, immunization of susceptible strains of rats or mice with MBP emulsified in incomplete Freund's adjuvant (IFA) was found to confer neuroprotection without causing disease development. We further show that the same T cell population participates both in the manifestation of autoimmune disease symptoms and in beneficial autoimmunity. However, the passive transfer of autoimmune T cells (Th1), in order to have a beneficial outcome, was found to require the participation of the injected Th1 cells directed to myelin antigens and of an additional CD4⁺ T cell population of the recipient. Furthermore, the injected Th1 cells in order to display neuroprotection should be viable autoreactive T cells, as demonstrated by the failure of injection of lethally irradiated T cells to improve neuronal survival.

2. Materials and methods

2.1. Animals

Inbred adult female Lewis and Sprague–Dawley (SPD) rats, adult wild-type C57Bl/6J and Balb/c mice, and transgenic C57Bl/6J *nu/nu* mice (devoid of mature T cells) were supplied by the Animal Breeding Center of The Weizmann Institute of Science. Animals were handled according to the regulations formulated by IACUC (Institutional Animal Care and Use Committee).

2.2. Antigens

MBP from guinea pig spinal cord and ovalbumin (OVA) were purchased from Sigma (St. Louis, MO). MBP peptides MBP 51–70 (APKRGCGKDSHTRTTHYG) and MBP 87–99 (VHFFKNIVTPRTP) were synthesized using 9-fluorenylmethoxycarbonyl technique with an automatic multiple peptide synthesizer (AMS422; ABIMED, Langenfeld, Germany) at the Weizmann Institute of Science. The encephalitogenic peptide myelin oligodendrocyte glycoprotein (MOG) 39–48 was synthesized at The Weizmann Institute of Science.

2.3. Antibodies

Mouse anti-rat CD4 antibodies conjugated to phycoerythrin and mouse anti-rat CD8 antibodies conjugated to fluorescein isothiocyanate were purchased from Serotec (Oxford, UK).

2.4. Optic nerve crush in rats and mice

The optic nerve was crushed as previously described in detail (Yoles and Schwartz, 1998). Using a binocular operating microscope, we anesthetized the animals and

exposed their right optic nerves. In rats, we used calibrated cross-action forceps to inflict a moderate or severe crush injury on the optic nerve, 1–2 mm from the eye. The severity of the injury determines the number of directly injured neurons. To assess neuroprotection, we inflicted a moderate crush injury on the optic nerve in Lewis rats (severe crush in this strain leaves almost no viable retinal ganglion cells (RGCs) because of poor endogenous neuroprotection) and a severe crush in SPD rats. To assess systemic and local inflammatory effects, we inflicted a severe crush in both strains. In mice, we inflicted a severe crush injury on the intraorbital portion of one optic nerve, leaving the contralateral nerve undisturbed.

2.5. Measurement of secondary neuronal degeneration in rats

Secondary degeneration of optic nerve axons was assessed by retrograde labeling of RGCs. This was done by the application, 2 weeks after crush injury, of the fluorescent lipophilic dye 4-(4-(didecylamino)styryl)-*N*-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes Europe, Leiden, The Netherlands) distally to the site of lesion, as previously described (Yoles and Schwartz, 1998).

2.6. Retrograde labeling of retinal ganglion cells in mice

This procedure was performed as previously described (Schori et al., 2001). The neurotracer dye FluoroGold (5% solution in saline; Fluorochrome, Denver, CO) was injected into the anesthetized mouse (1 μ l at a rate of 0.5 μ l/min in each hemisphere) using a Hamilton syringe, at a depth of 2 mm from the exposed brain surface, 2.92 mm posterior to the bregma and 0.5 mm lateral to the midline. One week after crush injury, the mice were killed and their retinas were detached and prepared as flattened whole mounts in 4% paraformaldehyde solution. Labeled cells from four to six selected fields of identical size (0.7 mm²) were counted.

2.7. Enzyme-linked immunosorbent assay

Anti-MBP T cells were grown for 1 week in a propagation medium, then washed with phosphate-buffered saline (PBS) and resuspended in stimulation medium. The T cells (0.5×10^6 cells/ml) were incubated, in the presence of irradiated thymocytes (10^7 cells/ml), with ConA (1.25 μ g/ml), or with MBP antigen (10 μ g/ml), or with no antigen, in stimulation medium at 37 °C, 98% relative humidity and 10% CO₂. After 48 h, the cells were centrifuged and their supernatants were collected and sampled according to the standard sandwich ELISA protocol for IFN- γ and IL-10 (R&D Systems, Minneapolis, MN). The plates were developed using a 3,3',5,5'-tetramethyl-benzidine liquid substrate system (Sigma). The reaction was stopped by addition of 1 M H₃PO₄, and the optical density was determined at 450 nm.

2.8. T cell lines

T cell lines were generated from draining lymph node cells obtained from Lewis rats immunized with the relevant antigens, as previously described (Moalem et al., 1999).

2.9. FACS analysis of CD4⁺ and CD8⁺ T cells

Cells were immunostained according to the manufacturer's instructions and were resuspended in 0.4 ml of 1% paraformaldehyde and analyzed by FACSsort (Becton-Dickinson), with 10,000 events scored. In single-color analysis, positive cells were defined as cells with higher immunofluorescence values, on a logarithmic scale, than those of control cells incubated with isotype antibodies as a control. The cells were scored from a region defined according to physical parameters that indicate the size (forward scatter) and granularity (side scatter) of lymphocytes, and were gated for staining with anti-CD4 and anti-CD8 antibodies.

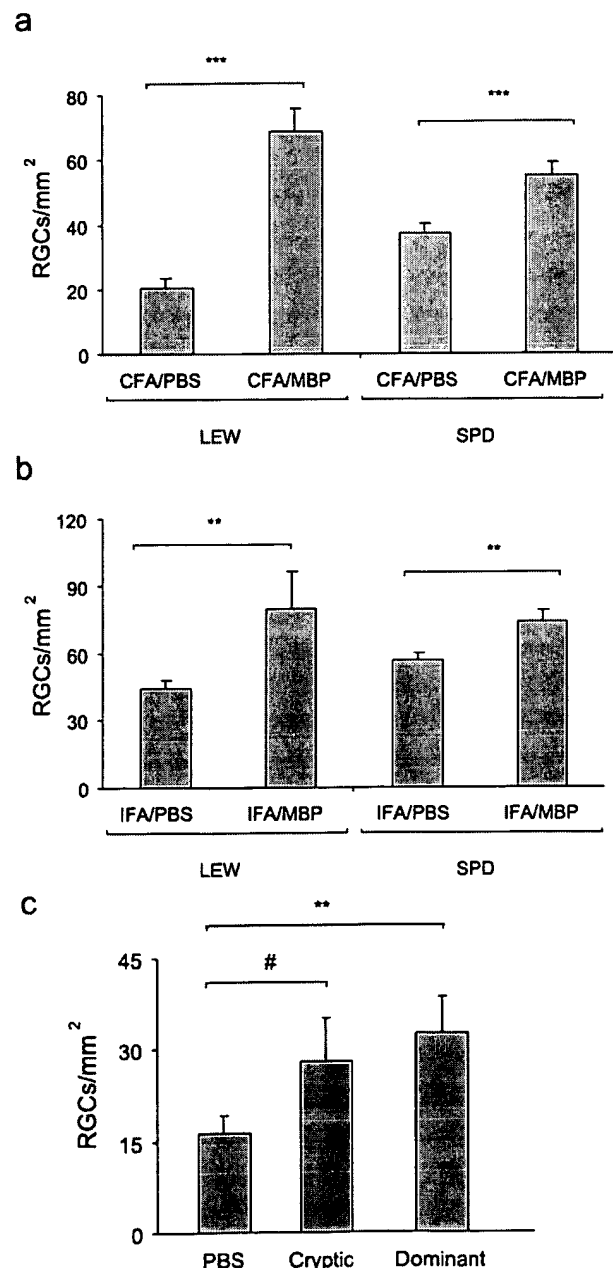
2.10. Preparation of splenocytes

Donor splenocytes from rats (aged up to 10 weeks) were obtained by rupturing the spleen and following conventional procedures. The splenocytes were washed with double-distilled water to eliminate red blood cells. Cells were run on a gradient of LSM® (Cappel, Aurora, OH) according to the manufacturer's instructions. Cells from the interphase were washed with PBS and counted.

2.11. Enrichment of mouse CD-positive T cells from splenocytes

CD4⁺ T cells were purified using the StemSep™ method for separation of murine cells (StemCell Technologies, Vancouver, BC, Canada). Splenocytes were isolated as described above and incubated with monoclonal antibodies CD11b (MAC-1), CD45R (B220), and CD8, myeloid differentiation antigen (Gr-1), and erythroid cells (TER119). This "depletion cocktail" is tailored for enrichment of CD4⁺ T cells (we achieved a purity of ~80%). Cells were separated using the standard StemCell™ protocol, using a magnetic

Fig. 1. Active immunization with MBP or MBP-derived peptides has a neuroprotective effect in both susceptible and resistant rat strains: susceptible Lewis and resistant SPD rats were immunized with 100 µg of MBP or with PBS emulsified in (a) complete Freund's adjuvant (CFA) or (b) incomplete Freund's adjuvant (IFA), 1 week before being subjected unilaterally to severe (a) or moderate (b) optic nerve crush injury ($n=10-12$ rats in each group). (c) Lewis rats were immunized with 100 µg of a cryptic (51–70) or a dominant (87–99) epitope of MBP, or with PBS emulsified in CFA, 1 week before being subjected unilaterally to severe optic nerve crush ($n=10-12$ rats in each group). To assess secondary degeneration, 2 weeks after injury, we applied the neurotracer dye 4-Di-10-Asp distally to the injury site, and 5 days later the rats were killed and their retinas were excised and flat-mounted. Labeled RGCs (representing surviving fibers), from four fields located at approximately the same distance from the optic disk in each retina, were counted under the fluorescence microscope. The neuroprotective effect (assessed by the number of neurons that survived the injury) of prior immunization with MBP emulsified in CFA (a) or IFA (b) was significantly greater than that of injection with PBS in the susceptible Lewis rats ($P<0.001$ or $P<0.01$, respectively, Student's *t*-test) as well as in the resistant SPD rats ($P<0.001$; $P<0.01$ respectively). The neuroprotective effect of prior immunization with the dominant epitope emulsified in CFA (c) was significantly greater than that of injection with PBS ($P<0.01$; Student's *t*-test) whereas immunization with the cryptic epitope was significant relative to PBS injection only when tested by a one-tailed Student's *t*-test ($P=0.082$ and $P=0.041$ for two-tailed and one-tailed Student's *t*-test, respectively).



cell depletion technique. Recovered cells were not labeled with antibody.

2.12. *In vitro* proliferative response of splenocytes

Splenocytes prepared from treated rats (quadruplicate samples for each rat) were assayed in 96-well plates. Each well contained 5×10^5 cells and antigens, as described in Results. When T cells were to be used as antigens, they were lethally irradiated (2000 rad) before use. The cells were cultured in 0.1 ml of proliferation medium for 48 h at 37 °C in humidified air with 7.5% CO₂. Cultures were pulsed with [³H]-thymidine for the last 18 h and then harvested by a multi-harvester on fiberglass filters. Thymidine incorporation was measured in a liquid scintillation counter.

2.13. Clinical score for experimental autoimmune disease

Symptoms of EAE were evaluated and graded on a 5-point scale: 0.5, loss of tail tonus; 1, tail paralysis; 2, gait disturbance; 3, hind limb paralysis; 4, tetraparesis; 5, death.

3. Results

3.1. Active immunization with myelin basic protein emulsified in incomplete or complete Freund's adjuvant protects neurons from secondary degeneration

Previous studies in our laboratory have shown that autoimmune T cells reactive to MBP confer neuroprotection when injected systemically into recipients with a CNS injury (optic nerve crush or spinal cord contusion), despite the fact that the treated animals also develop the transient autoimmune disease EAE. Here we show that neuroprotection can also be induced by active immunization of EAE-susceptible (Lewis) rats with MBP emulsified in complete Freund's adjuvant (CFA). Similar active immunization of the EAE-resistant SPD rats conferred neuroprotection (Fig. 1a). To verify that it is possible to obtain neuroprotection without inducing EAE in Lewis rats, we immunized rats of both strains with MBP emulsified in incomplete Freund's adjuvant (IFA). In both cases, a neuroprotective effect, without EAE development, was obtained (Fig. 1b).

We further examined whether treatment efficacy would be affected by the nature of the myelin-derived peptide. A neuroprotective autoimmune response was demonstrated in Lewis rats immunized with the cryptic epitope (51–70) of MBP, but the response was not as strong as that in rats immunized with the MBP dominant epitope (87–99). The latter group of rats, despite developing severe autoimmune disease, derived significant benefit from the vaccination (Fig. 1c).

3.2. Autoimmune Th1 cells confer neuroprotection on damaged neurons only when they act in conjunction with additional CD4-positive T cells

We first examined whether the cells that are neuroprotective in EAE-susceptible recipients are the injected anti-MBP T cells themselves or endogenous cells evoked in response to the injection.

Normal adult Lewis rats (an EAE-susceptible strain) and adult Lewis rats in which the thymus had been excised at birth were subjected to unilateral moderate optic nerve crush, followed immediately by passive transfer of autoimmune anti-MBP helper T cells (mostly Th1 cells), a population previously shown to be encephalitogenic (Moalem et al., 1999). Unlike the normal rats, the neonatally thymectomized rats received no benefit from the transferred of the anti-MBP helper T cells: the numbers of their RGCs per square millimeter (mean \pm SEM) were 48 ± 8 after the T cell transfer and 45 ± 3 without the transferred T cells (Fig. 2a). They did, however, develop EAE (Fig. 2b), as the transferred line was encephalitogenic, CD4⁺, reactive to MBP, and mostly of the Th1 phenotype, especially upon activation (Fig. 2c–e). When we transferred the MBP-reactive T cells into thymectomized Lewis rats along with an enriched population of CD4⁺ T cells (90–95% pure; isolated from the spleens of naïve Lewis rats; Fig. 2f) a significant neuroprotection following optic nerve crush in these rats was observed (Fig. 2g). These results thus suggest that a CD4 population of the recipient is involved in the process of the immune neuroprotection in addition to the transferred Th1 cells.

The encephalitogenic T cells used in our experiments were not cloned, but were derived from a rat primary T cell line which expresses a cytokine profile reminiscent of Th1/Th0 (Moalem et al., 2000a) (Fig. 2). To verify our assumption that the same T cell population is active in both autoimmune disease induction and immune neuroprotection, we conducted a similar experiment in mice, using the EAE-susceptible C57Bl/6J strain and cloned Th1 cells specifically directed to the dominant epitope (35–55) of the myelin-associated antigen MOG (Fig. 3a). An examination of the cytokine content of the Th1 clones showed that these T cells produce large quantities of IFN- γ but no detectable levels of IL-4 (Mizrahi et al., manuscript in preparation). Passive transfer of these homologous anti-MOG Th1 cells into wild-type C57Bl/6J mice after optic nerve injury was beneficial, resulting in the survival of significantly more RGCs than in untreated matched controls (806 ± 48 compared to 599 ± 87 ; $p < 0.05$; Fig. 3b). No such beneficial effect was obtained when the same cells were injected into nude (*nu/nu*) C57Bl/6J mice lacking mature T cells (398 ± 55 compared to 419 ± 55 in the controls). These results provide further evidence that the same population of T cells participates in both the beneficial (neuroprotective) and the destructive (disease-inducing) autoimmune response. They also lend further support to our contention

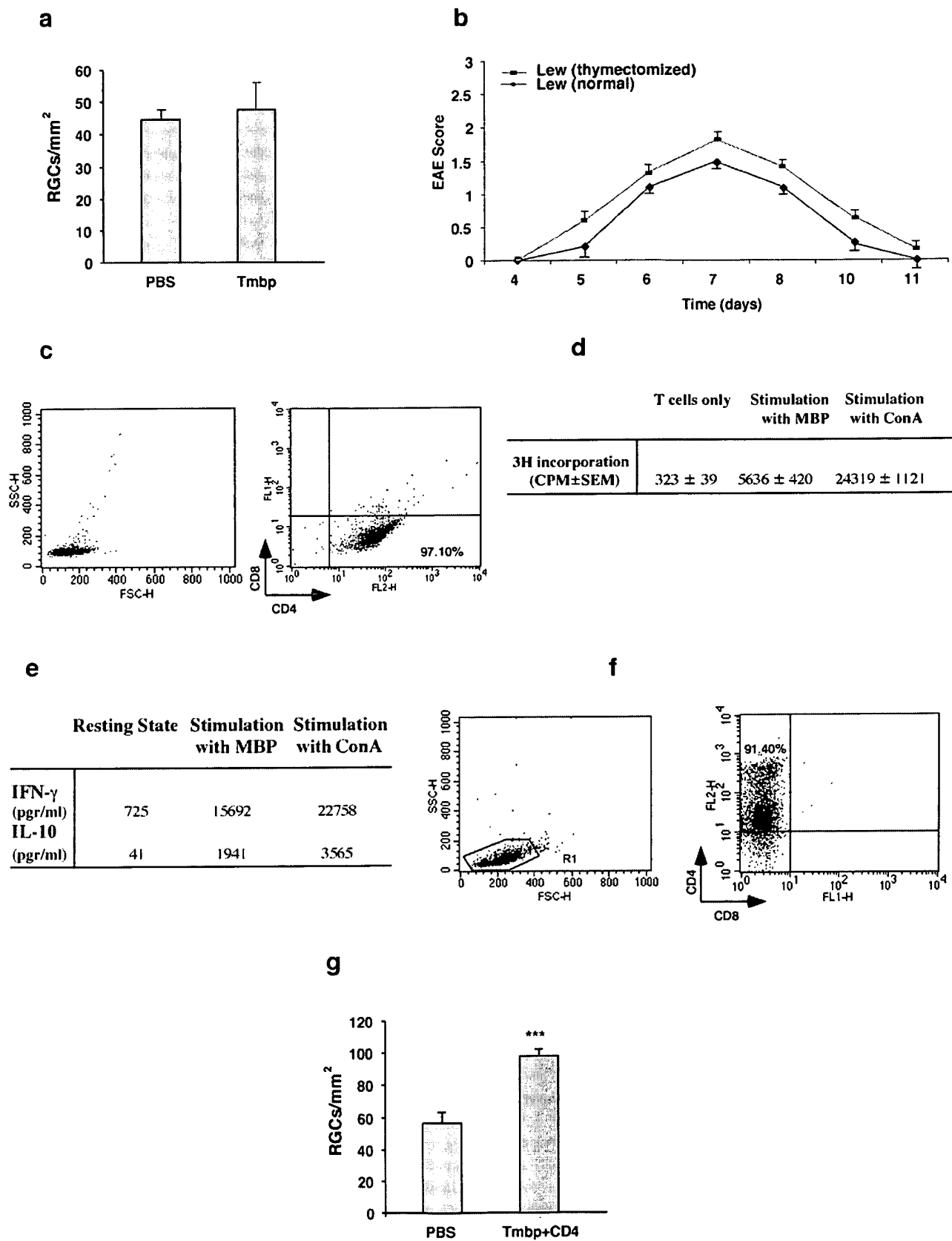


Fig. 2.

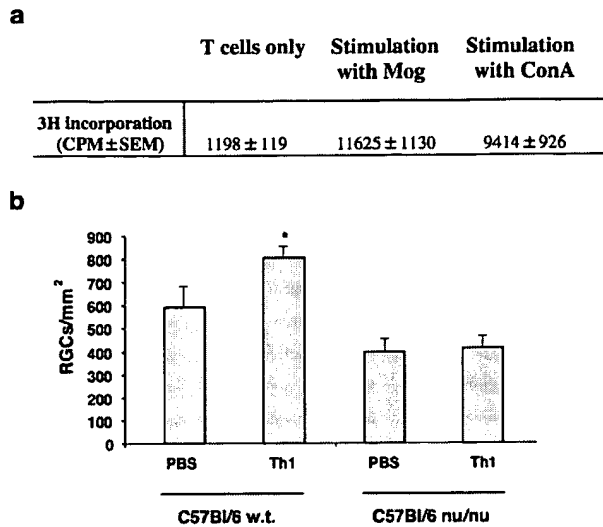


Fig. 3. The transferred autoimmune T cells responsible for evoking neuroprotection are Th1 cells: (a) Immediately after optic nerve crush injury, adult C57Bl/6J wild-type mice and C57Bl/6J *nu/nu* mice (devoid of T cells) were injected with a Th1 clone directed against a MOG-derived peptide. Injection of T cells into the wild-type mice resulted in increased neuronal survival ($n=5$ in each group; $P<0.05$; Student's *t*-test) relative to that in control mice injected with PBS only. Similar injection of Th1 cells into the *nu/nu* mice had no effect. (b) Thymidine incorporation assay of the T cells shows a strong antigenic specificity of this clone, compared to non-antigen mitogenic activation.

that protective autoimmunity requires the participation of myelin-specific Th1 cells. It appears, however, that expression of the beneficial effect of the injected Th1 cells in addition to or instead of destruction, requires an additional mechanism, supplied by a T cell population that is missing in the nude mice.

3.3. Passive transfer of non-viable T cells fails to promote neuronal survival

The finding that passively transferred myelin-specific autoimmune T cells are beneficial in rats and mice only when $CD4^+$ T cells are present in the recipients prompted us to examine whether the transferred cells are themselves the active players inducing protection, or are needed to evoke a T cell-dependent protective mechanism in the recipient. To address this question, we injected the optic nerve-injured Lewis rats with encephalitogenic autoimmune T cells that had been lethally irradiated. Matched

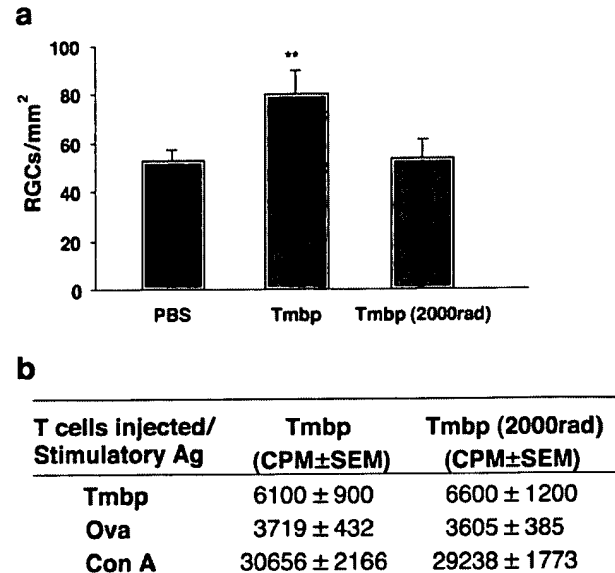


Fig. 4. T cells reactive to myelin basic protein, if not viable, lose their neuroprotective effect; T cell vaccination with lethally irradiated cells is not sufficient to induce neuroprotection: (a) Injection of viable autologous anti-MBP T cells into Lewis rats increased neuronal survival after optic nerve injury (Student's *t*-test; $P<0.01$ compared with PBS injection), whereas injection of lethally irradiated anti-MBP T cells had no such effect ($n=8-10$ in each group, Student's *t*-test; $p>0.5$). (b) Injection of irradiated and of viable T cell lines induced a similar T cell response, measured by a proliferation assay specific to the injected T cells (anti-idiotypic response) ($n=8-10$ for each T cell treatment).

injured Lewis rats injected with non-irradiated encephalitogenic T cells served as a positive control. Injection of the irradiated cells into Lewis rats with an intact thymus failed to elicit a neuroprotective response (mean numbers of RGCs per square millimeter were 52 ± 4 without the injected T cells and 53 ± 8 after T cell transfer; Fig. 4a), in spite of the fact that this procedure evoked a proliferative response directed to the injected inactivated T cells, similar to that evoked by injection of viable T cells (Fig. 4b) (Borghans et al., 1998).

4. Discussion

In this study, we showed that Th1 cells are needed for expression of the beneficial effect of autoimmunity after axonal injury in the CNS. We further showed that active immunization with an encephalitogenic myelin-related self-

Fig. 2. The transferred autoimmune T cells reactive to myelin proteins are necessary but not sufficient for immune neuroprotection: the recipient $CD4^+$ T cells are also required: (a) Anti-MBP T cells (mostly Th1 cells) injected immediately after optic nerve crush injury in adult Lewis rats, though strongly neuroprotective in normal rats, had no effect if the rats had been thymectomized at birth and therefore were devoid of endogenous T cells ($n=8-10$ in each group; Student's *t*-test; $P>0.5$). (b) The injected anti-MBP T cells were examined for expression of CD4 and CD8 surface markers by FACS (c) examined for expression of cytokines (IFN- γ and IL-10), and (d) assayed for thymidine incorporation to ensure antigen-specific activity. (e) The thymectomized recipients developed a monophasic EAE, similar to that seen in normal rats upon injection of the anti-MBP T cells. (f) Co-transfer of autoreactive T cells and naïve $CD4^+$ T cells restored the neuroprotective effect ($n=6-8$ in each group; $P<0.001$; Student's *t*-test). $CD4^+$ T cells were purified as described in Materials and methods. Their purity was found to range from 90% to 95% (a representative level of purification is presented (g)).

antigen has a beneficial effect on neuronal survival in rats after CNS injury (optic nerve crush). It appears that the requirements for neuroprotection and for induction of an autoimmune disease (EAE) are not the same, even though the activity of myelin-specific Th1 cells is common to both. Neuroprotection could be achieved under conditions (immunization with IFA) that did not allow EAE to develop, but at the same time a strong autoreactive response (obtained with a dominant epitope but not with a cryptic epitope) was needed for neuroprotection. For autoimmune disease induction the transfer of effector Th1 cells alone is sufficient, whereas for neuroprotection both the effector Th1 cells and another population of T cells, namely CD4⁺ cells that are endogenous to the recipient, are required.

Previous studies in our laboratory, showing that autoimmune T cells reactive to MBP confer neuroprotection on the damaged optic nerve, raised a fundamental question: is an active autoimmune activity required for neuroprotection or do the injected autoimmune T cells increase the regulatory network represented in part by anti-idiotypic T cell activity? The results of the present study show that active immunization of EAE-susceptible rats with MBP, emulsified in either CFA or IFA, has a strong neuroprotective effect. Since an autoimmune disease is not induced with IFA as emulsifier, it follows that autoimmune disease development is not a prerequisite for manifestation of protective autoimmunity. Interestingly, however, rats immunized with a dominant epitope of MBP showed significantly better neuronal survival than rats immunized with a cryptic MBP epitope, suggesting that neuroprotection probably requires the presence of a critical number of potent autoreactive T cells. These findings, together with others (Butovsky et al., 2001; Hammarberg et al., 2000a,b), raised questions about the relationship between the T cells that confer neuroprotection and those that cause a disease. Are they the same or different, and does either of these T cell populations need the participation of other cells for its operation?

An earlier study from our laboratory showed that after CNS injury in an EAE-susceptible (Lewis) strain, passive transfer of encephalitogenic T cells specific to myelin-associated antigens, in spite of inducing EAE, protects the damaged nerve from secondary neuronal degeneration (Hauben et al., 2000a; Moalem et al., 1999). In the present work the same cells, when injected into Lewis recipients devoid of mature T cells (due to neonatal thymectomy), were found to induce EAE, but without any neuroprotective benefit to the injured neural tissue. When these autoimmune T cells were co-injected with homologous purified CD4⁺ T cells into the thymectomized Lewis recipients, their beneficial effect was restored. No neuroprotection was observed, however, if the transferred cells were lethally irradiated, although the transferred cells were still capable of inducing a proliferative T cell response similar to that obtained with live transferred T cells. We conclude that passive transfer of live autoimmune T cells can beneficially affect the survival of injured neural tissue provided that an additional T cell

population (CD4⁺), endogenous to the recipient, is also present. The nature of this population and their role in protective autoimmunity is currently under investigation.

The results of this study further show that the beneficial outcome of an autoimmune T cell response is mediated by Th1 cells. Injection of Th2 cells does not induce neuroprotection (Mizrahi et al., manuscript in preparation). It should be noted that immunization in the resistant strain of SPD rats causes the spontaneous autoimmune protective response to be boosted, whereas in the susceptible Lewis rats, in which such a response is not spontaneously manifested, such immunization causes its induction. The Th1 response is significantly stronger when the adjuvant used for immunization is CFA rather than IFA (Lenz et al., 1999; Shibaki and Katz, 2002; Zhang et al., 1999). This might explain why immunization with CFA has a much more potent neuroprotective effect than immunization with IFA. Immunization with IFA is known to evoke the Th2 phenotype. At an early stage after the immunization, however, it evokes a short-lived Th1 phenotype (Shibaki and Katz, 2002; Zhang et al., 1999), which is apparently sufficient to accommodate the neuroprotective activity of Th1 cells within the therapeutic window of an acute insult. By the time the response shifts to evoking Th2, the relevant therapeutic window would in any case no longer be open. This might explain the efficacy of immunization with IFA.

We suggest that the activity of the Th1 cells in neural protection (i.e., in their response to self-antigens) does not differ from the activity of helper Th1 cells in their response to nonself antigens, i.e., providing well-controlled assistance to macrophages or other phagocytic or antigen-presenting cells in their activity of removing a perceived threat to the tissue by amplifying the innate response (Shaked et al., unpublished observations).

According to this view, the difference between beneficial autoimmunity (neuroprotection) and potentially destructive autoimmunity (autoimmune disease) derives not from the nature of the autoimmune T cells but from their regulation, which presumably affects such parameters as the time of onset, the strength, and the duration of the autoimmune response manifested at the site of a CNS insult. Therefore, contrary to the prevailing perception of autoimmunity as an accident of nature with consequences that are always harmful, we suggest that autoimmunity should instead be viewed as a desirable and beneficial response, though in need of proper regulation in order to fulfil its protective potential (Schwartz and Kipnis, 2001).

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References

- Borghans, J.A., De Boer, R.J., Sercarz, E., Kumar, V., 1998. T cell vaccination in experimental autoimmune encephalomyelitis: a mathematical model. *J. Immunol.* 161, 1087–1093.
- Butovsky, O., Hauben, E., Schwartz, M., 2001. Morphological aspects of spinal cord autoimmune neuroprotection: colocalization of T cells with B7-2 (CD86) and prevention of cyst formation. *Faseb J.* 15, 1065–1067.
- Cohen, I.R., Schwartz, M., 1999. Autoimmune maintenance and neuroprotection of the central nervous system. *J. Neuroimmunol.* 100, 111–114.
- Hafner, D.A., Weiner, H.L., 1987. T cells in multiple sclerosis and inflammatory central nervous system diseases. *Immunol. Rev.* 100, 307–332.
- Hammarberg, H., Lidman, O., Lundberg, C., Eltayeb, S.Y., Gielen, A.W., Muhallab, S., Svenningsson, A., Linda, H., van Der Meide, P.H., Cullheim, S., Olsson, T., Piehl, F., 2000a. Neuroprotection by encephalomyelitis: rescue of mechanically injured neurons and neurotrophin production by CNS-infiltrating T and natural killer cells. *J. Neurosci.* 20, 5283–5291.
- Hammarberg, H., Piehl, F., Risling, M., Cullheim, S., 2000b. Differential regulation of trophic factor receptor mRNAs in spinal motoneurons after sciatic nerve transection and ventral root avulsion in the rat. *J. Comp. Neurol.* 426, 587–601.
- Hauben, E., Butovsky, O., Nevo, U., Yoles, E., Moalem, G., Agranov, G., Mor, F., Leibowitz-Amit, R., Pevsner, E., Akselrod, S., Neeman, M., Cohen, I.R., Schwartz, M., 2000a. Passive or active immunization with myelin basic protein promotes recovery from spinal cord contusion. *J. Neurosci.* 20, 6421–6430.
- Hauben, E., Nevo, U., Yoles, E., Moalem, G., Agranov, E., Mor, F., Akselrod, S., Neeman, M., Cohen, I.R., Schwartz, M., 2000b. Autoimmune T cells as potential neuroprotective therapy for spinal cord injury. *Lancet* 355, 286–287.
- Hohlfeld, R., Kerschensteiner, M., Stadelmann, C., Lassmann, H., Wekerle, H., 2000. The neuroprotective effect of inflammation: implications for the therapy of multiple sclerosis. *J. Neuroimmunol.* 107, 161–166.
- Kipnis, J., Yoles, E., Porat, Z., Cohen, A., Mor, F., Sela, M., Cohen, I.R., Schwartz, M., 2000. T cell immunity to copolymer 1 confers neuroprotection on the damaged optic nerve: possible therapy for optic neuropathies. *Proc. Natl. Acad. Sci. U. S. A.* 97, 7446–7451.
- Kipnis, J., Yoles, E., Schori, H., Hauben, E., Shaked, I., Schwartz, M., 2001. Neuronal survival after CNS insult is determined by a genetically encoded autoimmune response. *J. Neurosci.* 21, 4564–4571.
- Lenz, D.C., Wolf, N.A., Swanborg, R.H., 1999. Strain variation in autoimmunity: attempted tolerization of DA rats results in the induction of experimental autoimmune encephalomyelitis. *J. Immunol.* 163, 1763–1768.
- Lundberg, C., Lidman, O., Holmdahl, R., Olsson, T., Piehl, F., 2001. Neurodegeneration and glial activation patterns after mechanical nerve injury are differentially regulated by non-MHC genes in congenic inbred rat strains. *J. Comp. Neurol.* 431, 75–87.
- Moalem, G., Leibowitz-Amit, R., Yoles, E., Mor, F., Cohen, I.R., Schwartz, M., 1999. Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nat. Med.* 5, 49–55.
- Moalem, G., Gdalyahu, A., Shani, Y., Otten, U., Lazarovici, P., Cohen, I.R., Schwartz, M., 2000a. Production of neurotrophins by activated T cells: implications for neuroprotective autoimmunity. *J. Autoimmun.* 20, 6421–6430.
- Moalem, G., Yoles, E., Leibowitz-Amit, R., Muller-Gilgor, S., Mor, F., Cohen, I.R., Schwartz, M., 2000b. Autoimmune T cells retard the loss of function in injured rat optic nerves [In Process Citation]. *J. Neuroimmunol.* 106, 189–197.
- Schori, H., Yoles, E., Schwartz, M., 2001. T-cell-based immunity counteracts the potential toxicity of glutamate in the central nervous system. *J. Neuroimmunol.* 119, 199–204.
- Schwartz, M., Kipnis, J., 2001. Protective autoimmunity: regulation and prospects for vaccination after brain and spinal cord injuries. *Trends Mol. Med.* 7, 252–258.
- Shibaki, A., Katz, S.I., 2002. Induction of skewed Th1/Th2 T-cell differentiation via subcutaneous immunization with Freund's adjuvant. *Exp. Dermatol.* 11, 126–134.
- Steinman, L., 2001. Multiple sclerosis: a two-stage disease. *Nat. Immunol.* 2, 762–764.
- Wekerle, H., 1998. Immune pathogenesis of multiple sclerosis. Brain auto-immune reactivity and its control by neuronal function. *Mult. Scler.* 4, 136–137.
- Yoles, E., Schwartz, M., 1998. Degeneration of spared axons following partial white matter lesion: implications for optic nerve neuropathies. *Exp. Neurol.* 153, 1–7.
- Yoles, E., Hauben, E., Palgi, O., Agranov, E., Gothilf, A., Cohen, A., Kuchroo, V., Cohen, I.R., Weiner, H., Schwartz, M., 2001. Protective autoimmunity is a physiological response to CNS trauma. *J. Neurosci.* 21, 3740–3748.
- Zhang, L., Mia, M.Y., Zheng, C.L., Hossain, M.A., Yamasaki, F., Tokunaga, O., Kohashi, O., 1999. The preventive effects of incomplete Freund's adjuvant and other vehicles on the development of adjuvant-induced arthritis in Lewis rats. *Immunology* 98, 267–272.

T cell immunity to copolymer 1 confers neuroprotection on the damaged optic nerve: Possible therapy for optic neuropathies

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Contributed by Michael Sela, April 12, 2000

We recently reported that the posttraumatic spread of degeneration in the damaged optic nerve can be attenuated by the adoptive transfer of autoimmune T cells specific to myelin basic protein. However, it would be desirable to obtain immune neuroprotection free of any possible autoimmune disease. In an attempt to obtain disease-free immune neuroprotection, we used the synthetic four-amino acid polymer copolymer 1 (Cop-1), which is known not to be encephalitogenic despite its cross-reactivity with myelin basic protein. We show here that active immunization with Cop-1 administered in adjuvant, as well as adoptive transfer of T cells reactive to Cop-1, can inhibit the progression of secondary degeneration after crush injury of the rat optic nerve. These results have implications for the treatment of optic neuropathies.

Optic neuropathies are neurodegenerative diseases of the optic nerve in which degeneration starts at the optic nerve and ends with the death of the retinal ganglion cells (RGCs) (1, 2). In many cases of optic neuropathies, the primary risk factors may be removed but degeneration continues. We have suggested that the progression of damage may be attributable to the fact that at any given time fibers undergoing acute degeneration create a hostile environment for neighboring fibers that are still intact, thereby causing their eventual degeneration (3, 4). This process of "secondary degeneration" is like that seen after any traumatic injury to other areas of the central nervous system (CNS), such as the spinal cord and the brain. Hence neuroprotection should be a useful complement to the treatment of optic neuropathies.

We recently demonstrated that the adoptive transfer of T cells specific to proteins associated with CNS myelin, such as myelin basic protein (MBP), can reduce the posttraumatic secondary degeneration of the rat optic nerve and spinal cord (refs. 5, 6 and 8 and G. Moalem, A. Gdalyahu, Y. Shani, U. Otten, P. Lazarovici, I.R.C., and M. S., unpublished work). We further showed that this neuroprotective effect is not restricted to T cells directed against major encephalitogenic peptides, because T cells against cryptic peptides in the MBP molecule were similarly effective in reducing secondary degeneration (6). However, in the development of therapies based on autoimmune neuroprotection, it is important to seek "safe" antigenic epitopes that will not cause the induction of an autoimmune disease.

The question was whether we could induce a protective immune response with a nonself protein resembling or having a cross-recognition with the self protein, but without the danger of disease induction. A possible candidate for this role is the nonpathogenic polymer, copolymer 1 (Cop-1, trade name Copaxone). Cop-1 is a synthetic amino acid polymer (4.7–11 kDa) composed of L-alanine, L-lysine, L-glutamic acid, and L-tyrosine, in a molar ratio of 4.2:3.4:1.4:1.0 (9). It initially was designed to mimic MBP and induce experimental autoimmune encephalomyelitis (EAE), but was found to be nonencephalitogenic and to suppress MBP-induced EAE. Cop-1 also blocks chronic-relapsing EAE induced by mouse spinal cord homogenate or by the encephalitogenic peptides of

proteolipid protein in a (SJL/J × BALB/c) F₁ mouse model (10). Cop-1 binds to the relevant major histocompatibility complex proteins and leads to the activation of T suppressor cells, which are triggered by determinants common to Cop-1 and MBP (11). We reasoned that the existence of cross-reaction between Cop-1 and MBP or other components of myelin might enable Cop-1-specific T cells to recognize the damaged tissue, accumulate there, and become activated to induce neuroprotection. We also were interested in finding out whether, in addition to adoptive transfer, active immunization with Cop-1 could be used to reinforce immune neuroprotection.

In the present study we show that neuroprotection of crush-injured rat optic nerves can be obtained by active immunization with Cop-1 on the day of injury and by adoptive transfer of Cop-1 reactive T cells.

Materials and Methods

Animals. Inbred female adult Lewis rats (8–12 weeks old) were supplied by the Animal Breeding Center of The Weizmann Institute of Science. The rats were housed in a light- and temperature-controlled room and matched for age in each experiment. Animals were handled according to the regulations formulated by the Institutional Animal Care and Use Committee.

Antigens. MBP from the spinal cords of guinea pigs and ovalbumin (OVA) were purchased from Sigma. Cop-1 was purchased from Teva Pharmaceuticals (Petach-Tikva, Israel).

Antibodies. Mouse mAbs specific to rat T cell receptor (TCR) were kindly provided by Boris Reizis, Israel. Cy-3-conjugated goat anti-mouse IgG (with minimal cross-reaction to rat, human, bovine, and horse serum proteins) was purchased from Jackson ImmunoResearch.

T Cell Lines. T cell lines were generated from draining lymph node cells obtained from Lewis rats immunized with the above antigens (12). The antigen was dissolved in PBS (1 mg/ml) and emulsified with an equal volume of incomplete Freund's adjuvant (IFA) (Difco) supplemented with 4 mg/ml *Mycobacterium tuberculosis* (Difco). Ten days after the antigen was injected into the rats' hind foot pads in 0.1 ml of the emulsion, the rats were killed and their draining lymph nodes were surgically removed and dissociated. The cells were washed and activated with the antigen (10 µg/ml) in stimulation medium containing DMEM

Abbreviations: BDNF, brain-derived neurotrophic factor; CFA, complete Freund's adjuvant; CNS, central nervous system; Cop-1, copolymer 1; 4-Di-10-Asp, 4-(4-(didecylamino)styryl)-N-methylpyridinium iodide; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; NT, neurotrophin; OVA, ovalbumin; RGC, retinal ganglion cells; TCR, T cell receptor.

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supplemented with L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), sodium pyruvate (1 mM), penicillin (100 units/ml), streptomycin (100 μ g/ml), nonessential amino acids (1 ml/100 ml), and autologous serum 1% (vol/vol). After incubation for 72 h at 37°C, 98% relative humidity and 10% CO₂, the cells were transferred to propagation medium consisting of DMEM, L-glutamine, 2-mercaptoethanol, sodium pyruvate, nonessential amino acids, and antibiotics in the same concentrations as above, with the addition of 10% FCS (vol/vol) and 10% T-cell growth factor derived from the supernatant of Con A-stimulated spleen cells (13). Cells were grown in propagation medium for 4–10 days before being restimulated with their antigen (10 μ g/ml) in the presence of irradiated (2,000 rad) thymus cells (10^7 cells/ml) in stimulation medium. The T cell lines were expanded by repeated stimulation and propagation (14).

Crush Injury of Optic Nerve. The optic nerve was subjected to crush injury as described (15). Briefly, rats were deeply anesthetized by i.p. injection of XYL-M 2% (xylazine, 10 mg/kg; Arendonk, Belgium) and Ketaset (ketamine, 50 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA). Using a binocular operating microscope, lateral canthotomy was performed in the right eye, and the conjunctiva was incised lateral to the cornea. After separation of the retractor bulbi muscles, the optic nerve was exposed intraorbitally by blunt dissection. Using calibrated cross-action forceps; the optic nerve was subjected to a crush injury 1–2 mm from the eye. Mild and severe crush injuries were inflicted for short-term trials (2 weeks), as this time period was shown to be optimal for demonstrating secondary degeneration and its response to treatment (16). The uninjured contralateral nerve was left undisturbed.

Measurement of Secondary Degeneration by Retrograde Labeling of RGCs. Secondary degeneration of the optic nerve axons and their attached RGCs was measured after postinjury application of the fluorescent lipophilic dye, 4-(4-(didecylamino)-styryl)-N-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes), distally to the lesion site, 2 weeks after crush injury. Because only axons that are intact can transport the dye back to their cell bodies, application of the dye distally to the lesion site after 2 weeks ensures that only axons that survived both the primary damage and the secondary degeneration will be counted. This approach enabled us to differentiate between neurons that are still functionally intact and neurons in which the axons are injured but the cell bodies are still viable, because only those neurons whose fibers are morphologically intact can take up dye applied distally to the site of injury and transport it to their cell bodies. Using this method, the number of labeled RGCs reliably reflects the number of still-functioning neurons. Labeling and measurement were carried out as follows: the right optic nerve was exposed for the second time, again without damaging the retinal blood supply. Complete axotomy was performed 1–2 mm from the distal border of the injury site and solid crystals (0.2–0.4 mm diameter) of 4-Di-10-Asp were deposited at the site of the newly formed axotomy. Five days after dye application the rats were killed. The retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined for labeled RGCs by fluorescence microscopy.

ELISA. Anti-MBP or anti-Cop-1 T cells were grown for a week in a propagation medium, then washed with PBS and resuspended in stimulation medium. The T cells (0.5×10^6 cells/ml) were incubated, in the presence of irradiated thymocytes (10^7 cells/ml), with Con A (1.25 μ g/ml), MBP antigen (10 μ g/ml), Cop-1 antigen (10 μ g/ml), OVA antigen (10 μ g/ml), or with no antigen, in stimulation medium at 37°C, 98% relative humidity and 10% CO₂. In addition, irradiated thymocytes

(10^7 cells/ml) alone were incubated in stimulation medium. After 48 h the cells were centrifuged and their supernatants were collected and sampled. Concentrations of neurotrophin (NT)-3, nerve growth factor, and NT-4/5 in the samples were determined by the use of sandwich ELISA kits (Promega) and comparison with a NT standard (absorbance measurement at 450 nm using an ELISA reader). Concentrations of brain-derived neurotrophic factor (BDNF) in the samples were determined with a sensitive sandwich ELISA. In brief, 96-well flat-bottomed plates were coated with a chicken anti-human BDNF antibody (Promega) in 0.025 M NaHCO₃ and 0.025 M Na₂CO₃ (pH 8.2). Recombinant human BDNF (used as standard; Research Diagnostics, Flanders, NJ) was used in serial dilutions in blocking solution containing 3% BSA, 0.05% polyoxyethylene-sorbitan monolaurate (Tween-20), and 1% FCS in PBS (pH 8.2). Bound BDNF was detected by incubating the plates with a mouse anti-human BDNF antibody (Research Diagnostics) and then with peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) in blocking solution. The plates were developed by using a 3,3',5,5'-tetramethylbenzidine liquid substrate system (Sigma). The reaction was stopped by adding 1 M H₃PO₄, and the optical density was determined at 450 nm. Results for each experiment were calculated as the amount of secreted NT per 1 ml of sample, after subtraction of the background levels of the irradiated thymocytes incubated with the stimulation medium.

Immunization. Lewis rats (8–12 weeks old) each were injected with a total of 100 μ g of Cop-1 emulsified with an equal volume of complete Freund's adjuvant (CFA) containing 5 mg/ml of mycobacteria H37 RA (Difco). The emulsion, in a total volume of 0.2 ml, was injected into the rat's two hind footpads immediately after crush injury to the optic nerve. Beginning 24 h after the injury, each rat was fed 1-mg of Cop-1 every 24 h for 5 days.

Immunohistochemistry. Longitudinal cryosections (10 μ m thick) of the nerves were picked up onto gelatin-coated glass slides and frozen until preparation for fluorescence staining. The sections were fixed in ethanol for 10 min at room temperature, washed twice with double-distilled water, and incubated for 3 min in PBS containing 0.05% Tween-20. Sections then were incubated for 1 h at room temperature with mouse anti-rat mAbs to TCR (17) diluted in PBS containing 3% FCS and 2% BSA. The sections then were washed three times with PBS containing 0.05% Tween-20 and incubated with Cy63-conjugated goat anti-mouse IgG (with minimal cross-reaction to rat, human, bovine, and horse serum proteins; Jackson ImmunoResearch) for 1 h at room temperature. The sections were washed with PBS containing Tween-20 and treated with glycerol containing 1,4-diazobicyclo-(2, 2, 2)octane to inhibit quenching of fluorescence. The sections were viewed with a Zeiss Universal fluorescence microscope.

Results

Adoptive Transfer of T Cells Reactive to Cop-1 Is Neuroprotective in the Injured Optic Nerve. To examine whether T cells to Cop-1 display a neuroprotective effect in the rat optic nerve model, we injected 10×10^6 Cop-1-reactive T cells into rats immediately after a mild or severe injury of their optic nerves. After 2 weeks, the number of surviving fibers was determined by applying the dye 4-Di-10-Asp distally to the lesion site. Retinas were excised 5 days later and whole-mounted, and labeled RGCs were counted. Rats injected with Cop-1-reactive T cells on the day of a mild or severe crush injury to the optic nerve showed significantly less secondary degeneration than that seen in control rats injected with PBS (Fig. 1). None of the rats in these experiments developed EAE (data not shown). Representative fields from retinas treated with Cop-1-reactive T cells and from untreated

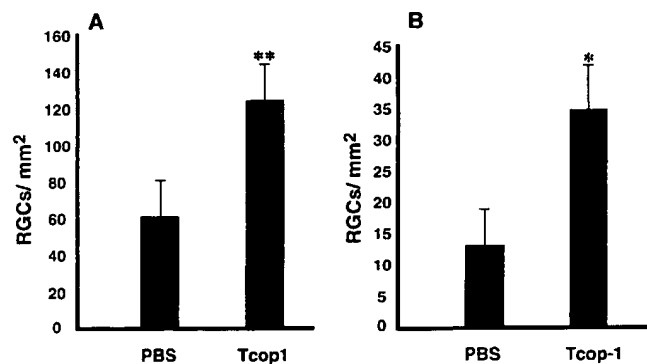


Fig. 1. T cells specific to Cop-1 protect neurons from secondary degeneration. Immediately after mild (A) or severe optic nerve injury (B), rats were injected with PBS or with Cop-1-specific T cells. For assessment of secondary degeneration, the neurotracer dye 4-Di-10-Asp was applied to the optic nerve distal to the site of injury, 2 weeks after the injury. After 5 days, the rats were killed and their retinas were excised and flat-mounted. Labeled (surviving) RGCs, from four fields located at approximately the same distance from the optic disk in each retina, were counted under a fluorescence microscope. The neuroprotective effect of Cop-1-reactive T cells compared with that of PBS was significant for both mild crush injury ($P < 0.005$, Student's *t* test) and severe crush injury ($P < 0.05$, Student's *t* test). The graph shows representative results of one experiment of the three that were performed with 6–10 animals in each group in each experiment.

retinas are shown in Fig. 2. It should be noted that rats injected with OVA-reactive T cells did not have any effect, as previously reported (6).

Cop-1-Reactive T Cells Accumulate in Both Injured and Uninjured Neuronal Tissues. To investigate whether T cells to Cop-1 accumulate at the site of the injury, we injected rats with Cop-1 reactive T cells and excised the nerves 7 days later, at the time point previously identified as the peak of accumulation of T cells in a damaged optic nerve (18, 19). The adoptive transfer of Cop-1-reactive T cells in the present study also caused a significant accumulation of T cells at the site of injury relative to the accumulation of T cells in the PBS-treated injured rats. T cell accumulation in the Cop-1-treated rats was greatest on day 7 after the injection (Fig. 3A). These findings were in line with our earlier results showing that injection of T cell lines of different specificities results in T cell accumulation at the site of the lesion.

In contrast to the accumulation of activated T cells at the site of injury after injection of T cells of any specificity, only T cells specific to CNS self-antigens were found to accumulate in uninjured nerves (18, 19). To learn whether Cop-1-specific T cells might behave like these anti-self T cells, we injected naive rats with T cells to MBP, OVA, or Cop-1, and excised the optic nerves 3 days later. Injection of T cells to Cop-1, like the injection of T cells to MBP (Fig. 3D), resulted in accumulation of T cells in the uninjured nerve (Fig. 3B). In contrast, the injection of T cells to OVA did not result in any detectable T cell accumulation in the uninjured optic nerve (Fig. 3C). Although there was less accumulation of T cells, in uninjured nerve, after injection of T cells reactive to Cop-1 than of T cells specific to MBP, these findings support the conclusion that Cop-1-reactive T cells can recognize *in vivo* an antigen in the intact optic nerve, presumably myelin-associated.

Cytokine and NT Profiles of T Cells to MBP or Cop-1. Anti-MBP T cells were stimulated *in vitro* with their specific antigen or Cop-1. The cultured media containing products secreted by these cells were collected and their cytokine contents were quantified by ELISA. The activated T cells secreted much larger amounts of cytokines than did the unstimulated T cells. The T helper 1-specific cytokine IFN- γ , was similarly expressed by both anti-MBP T cells and the anti-Cop-1 T cells, whereas the T helper 2-specific cytokine IL-10 was found to be secreted mainly by the anti-Cop-1 T cells. The largest amounts of secreted cytokines were detected in the supernatants of T cells stimulated with Con A (Table 1).

Up-regulation of neurotrophic expression and secretion by T cells activated with their specific antigens recently was demonstrated by our group (G. Moalem, A. Gdalyahu, Y. Shani, U. Otten, P. Lazarovici, I.R.C., and M. S., unpublished work and ref. 20). In an attempt to gain an insight into the mechanism underlying T cell-mediated neuroprotection, the T cell supernatants in the present study were subjected to ELISA to determine the NT profiles of T cells responsible for neuroprotection. The Cop-1-stimulated T cells secreted both nerve growth factor and NT-4/5, but in lower amounts than those secreted by the anti-MBP T cells. Relative to the production by anti-MBP T cells, the production of NT-3 by the Cop-1-stimulated T cells was insignificant; the production of BDNF, however, was massive (Fig. 4A). Thus, the Cop-1-stimulated T cells produced smaller amounts of all of the examined neurotrophic factors, with the notable exception of BDNF (Fig. 4A). Four independent determinations of the amounts of NT-3 and BDNF secreted by the differentially stimulated T cells yielded similar results. In each

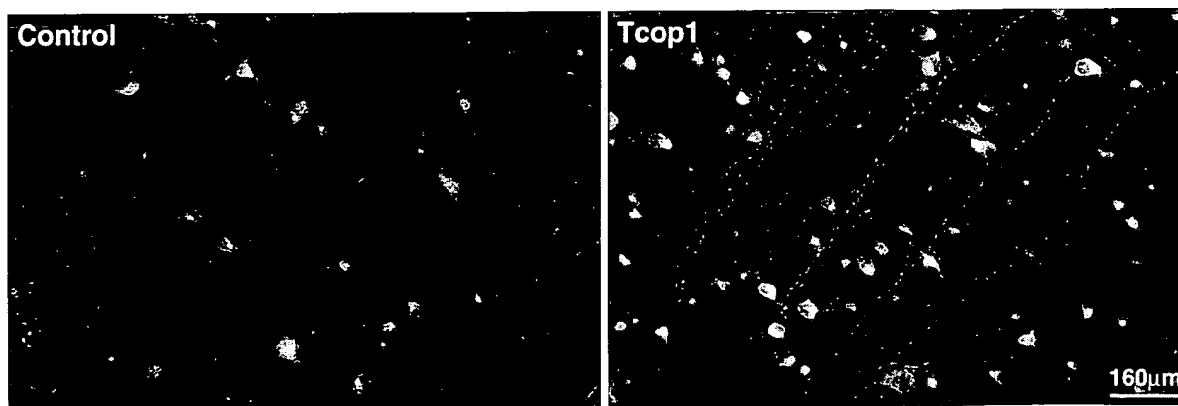


Fig. 2. Photomicrographs of retrogradely labeled retinas of injured optic nerves of rats. Immediately after unilateral crush injury of their optic nerves, rats were injected with Cop-1-reactive T cells or PBS. Two weeks later the neurotracer dye 4-Di-10-Asp was applied to the optic nerves distal to the site of injury, and 5 days later the retinas were excised and flat-mounted. Shown are representative fields with labeled RGCs, located at approximately the same distance from the optic disk in the retinas of the two groups.

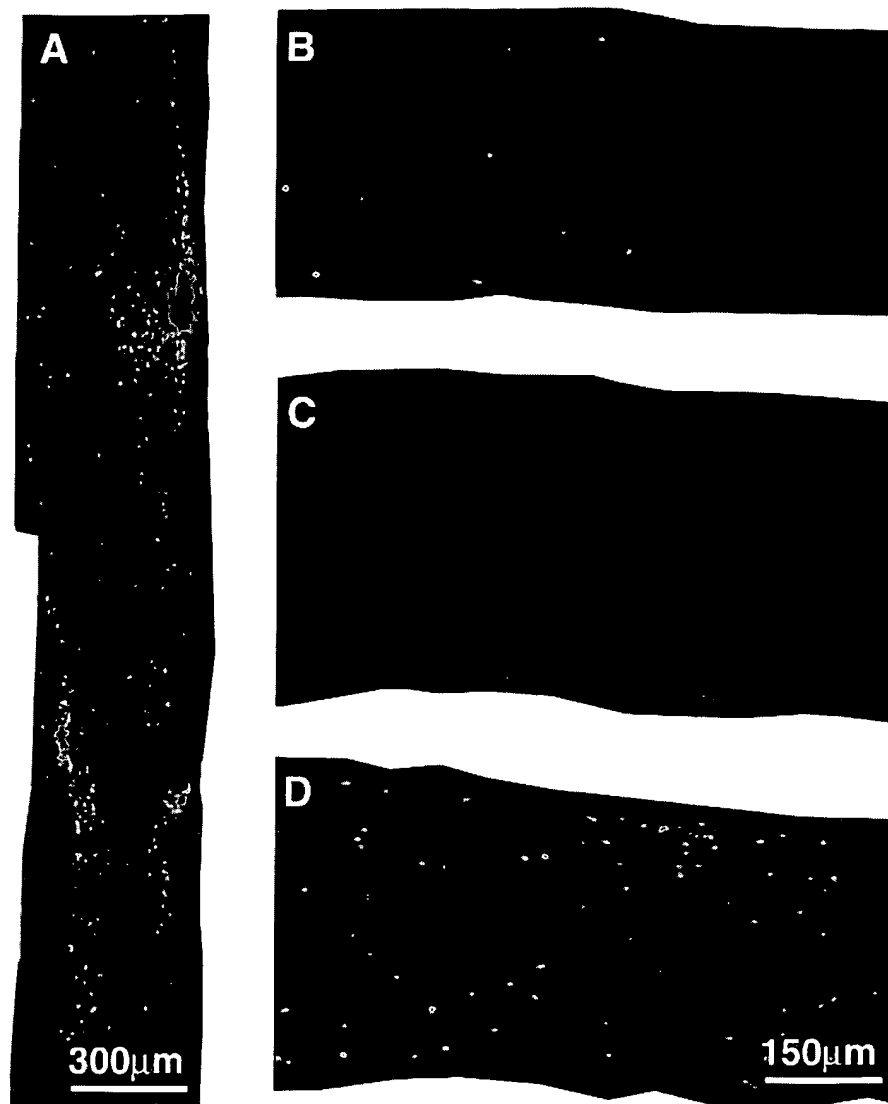


Fig. 3. *In situ* detection of immunostained T cells. (A) Injured nerve. (B–D) Uninjured nerves. (A) Seven days after crush injury and systemic injection of T cells reactive to Cop-1, cryosections (10 µm thick) of injured optic nerve were stained for TCR. Uninjured nerves were excised 7 days after injection with T cells specific to Cop-1 (B), OVA (C), or MBP (D), and then cryosectioned (10 µm) and stained for TCR. Representative slices are shown. At least four rats were tested in each experiment.

case, Cop-1-stimulated T cells produced about 2.5-fold more BDNF than anti-MBP T cells, and only 10% of the amounts of NT-3 (Fig. 4B).

Vaccination with Cop-1 Followed by Oral Administration of Cop-1 Protects Neurons from Secondary Degeneration. Oral administration of antigen in low doses is known to boost the immune

response and switch it toward T helper 2 regulatory cells, but in high doses it may cause anergy or depletion (21, 22). We postulated that if rats with crush-injured optic nerves were vaccinated with Cop-1 in CFA and then fed with low-dose Cop-1, the immune response to the vaccination might be boosted. Anesthetized rats were subjected to mild crush injury of the optic nerve, immediately vaccinated, and then fed for 5 days with

Table 1. ELISA of secreted cytokines

Cytokine, pgr/ml	Resting state		Stimulation with MBP		Stimulation with Cop-1		Stimulation with Con A	
	Tmbp	Tcop	Tmbp	Tcop	Tmbp	Tcop	Tmbp	Tcop
IFN- γ	725	6,645	15,692	925	7,242	11,825	22,758	22,525
IL-10	41	382	1,941	13	365	7,244	3,565	6,503

Supernatants from unstimulated T cells or T cells stimulated for 48 h with Con A mitogen, MBP antigen, or Cop-1 antigen in stimulation medium were subjected to sandwich ELISA. The table shows the concentration of cytokines. The amounts of secreted T helper 2-related cytokines were significantly higher in supernatants of Cop-1-reactive T cells than in supernatants of T cells specific to MBP.

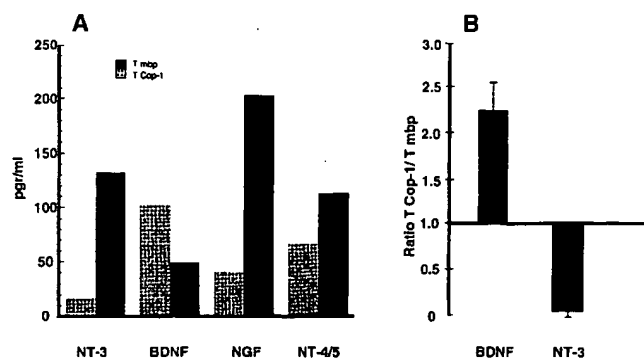


Fig. 4. ELISA of secreted neurotrophic factors. Rat anti-MBP or anti-Cop-1 T cells were cultured for 48 h with their specific antigen in stimulation medium. The T-cell supernatants were collected and subjected to sandwich ELISA. The histogram shows the concentration of secreted NTs in each sample. The amounts of nerve growth factor (NGF) and NT-4/5 were higher in the anti-MBP T cells than in the anti-Cop-1 T cells. NT-3 was found almost exclusively in T cells specific to MBP, whereas the amounts of BDNF secreted in the supernatants of Cop-1-stimulated T cells were significantly higher than in the supernatants of anti-MBP T cells. Representative data are shown (A). The mean ratios \pm SD (from five independent experiments) of the amounts of BDNF or NT-3 secreted by anti-Cop-1 T cells to the amounts secreted by anti-MBP T cells are shown in B.

Cop-1 dissolved in PBS. After 2 weeks the RGCs were retrogradely labeled and 5 days later the retinas were excised. Rats vaccinated with Cop-1 and then fed with the antigen showed evidence of significant neuroprotection compared with that in control rats injected with PBS in CFA and then fed with PBS (Fig. 5).

Discussion

The results of this study demonstrate a possible neuroprotective effect of T cell immunity to Cop-1 in a crush-injured CNS nerve. In the rat model of partial optic nerve crush, adoptive administration of Cop-1-reactive T cells or vaccination with Cop-1 on the day of CNS injury had a marked preventive effect on the secondary degeneration of nerve fibers.

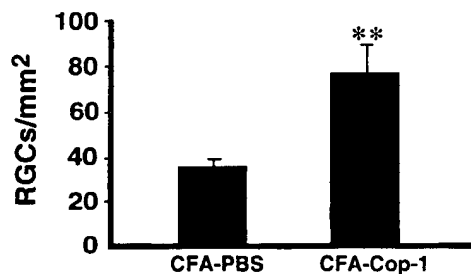


Fig. 5. Neuroprotection by active immunization with Cop-1. Immunization with Cop-1 in CFA, boosted with oral administration of the antigen, leads to protection against secondary degeneration. Immediately after mild optic nerve injury, rats were immunized s.c. with PBS in CFA or Cop-1 in CFA. For assessment of secondary degeneration, the neurotracer dye 4-Di-10-Asp was applied to the optic nerve distal to the site of injury 2 weeks after crush injury, and 5 days later the rats were killed and their retinas were excised and flat-mounted. Labeled (surviving) RGCs, from four fields located at approximately the same distance from the optic disk in each retina, were counted under the fluorescence microscope. The neuroprotective effect of Cop-1 immunization compared with that of PBS injection was significant ($P < 0.01$, Student's *t* test). The results are the summary of two experiments, each carried out with 5–6 rats in each group.

Cop-1 originally was designed to mimic MBP and to induce EAE, but was found to be nonencephalitogenic and even to suppress EAE induced by MBP (9), proteolipid protein (10), or myelin oligodendrocyte glycoprotein (23). The precise mechanisms by which Cop-1 prevents the development of EAE and ameliorates multiple sclerosis are not yet known. Nevertheless, some important immunological properties of this copolymer have emerged. Studies have demonstrated partial cross-reactivity of Cop-1 with MBP at both the T cell (24) and the antibody (25) level. Cop-1 can serve as an antagonist of the T-cell antigen receptor for the MBP immunodominant epitope (26). It also can bind to various major histocompatibility complex class II molecules and prevent them from binding to T cells with specific antigen-recognition properties (27). In rodents, Cop-1 induces regulatory cells that suppress the encephalitogenic T cells. Adoptive transfer of such T cells was found to prevent the development of EAE induced by MBP (28), proteolipid protein (10), or whole spinal cord homogenate (29).

T cells reactive to MBP were shown to be neuroprotective in rat models of partially crushed optic nerve (6) and spinal cord injury (8). The massive accumulation of exogenously administered T cells at the site of CNS injury suggests that the presence of T cells at the site of injury plays a prominent role in neuroprotection. It appears, however, that the accumulation, although a necessary condition, is not sufficient for conferring neuroprotection, as T cells specific to the non-self antigen OVA also accumulate at the site, but have no neuroprotective effect (18). As a feasible scenario, we suggest that T cells arriving at the site of injury recognize the antigen(s) presented there as major histocompatibility complex class II molecules (which are known to be up-regulated after CNS trauma) (19), and thus become activated, switching to a neuroprotective phenotype and producing factors that promote neuroprotection. It seems that recognition of TCR–major histocompatibility complex class II antigens at the site of injury is a precondition for activation of T cells and achievement of a neuroprotective effect.

The fact that T cells accumulate in the uninjured optic nerve supports the notion of recognition between the Cop-1-specific T cells and antigens presented by the optic nerve. It should be noted that the method used here to assess T cell accumulation involved the use of antibodies to the TCR. Using this approach, it is not possible to determine whether the accumulated T cells detected in the nerve are the ones that were injected. In our earlier studies we showed, however, that the injected activated T cells are indeed the ones that accumulate (18). Activated T cells can pass through the blood–brain barrier regardless of their specificity, but only those that are reactive to CNS antigens can accumulate in the uninjured nerve (30). Thus, our present findings demonstrate *in vivo* cross-recognition between Cop-1-reactive T cells and components of CNS myelin. This recognition probably serves as the trigger for T cell reactivation, causing the T cells to switch toward the protective phenotype. We found in this study that Cop-1-reactive T cells activated by their specific antigen secrete significant amounts of BDNF, an NT that plays a major role in neuronal survival (31, 32). It is possible that myelin proteins expressed at the lesion site reactivate the Cop-1-specific T cells to a similar extent.

Immunization with Cop-1, unlike immunization with MBP and other myelin-associated proteins, does not induce EAE, and the T cells evoked by Cop-1 in the absence of adjuvants are of a regulatory nature. In the present study, immunization with Cop-1 in CFA immediately after the injury, followed by five consecutive daily feedings, had a strongly neuroprotective effect. Such immunization is likely to evoke a cellular response that is large enough to exert at least some neuroprotective activity. It is possible that this response was somewhat delayed relative to the response obtained after adoptive transfer of T cells, but nevertheless it was still achieved within the time window needed for

protection of nerve fibers that escaped the primary lesion. Previous studies in the rat optic nerve have shown that the loss of neurons resulting from secondary degeneration is about 25% a week after mild crush injury and about 55% 2 weeks after the injury (16). Thus, even if the T cell response took 1 week to reach the required strength, there would still be nerve fibers in need of protection at that time. A comparison of our results obtained after adoptive transfer of activated Cop-1-reactive T cells and after active immunization with Cop-1 shows that the extent of protection from secondary degeneration was almost the same in both. It is worth noticing that, although Cop-1 is known as an agent designed to suppress T cell autoimmunity, its effect in this study requires that an anti-myelin T cell autoimmunity be activated. Thus, Cop-1 is used here as a potentially "safe" antigen for activating an effective T cell response that can be cross-activated by myelin proteins.

In conclusion, earlier studies have proposed that axonal injury in the rat CNS awakens an autoimmune T cell response which is directed against myelin proteins, but is too weak to protect the nerve fibers from secondary degeneration (6, 33). Boosting of this immune response without risk of accompanying autoimmune disease was achieved in this study by using

a copolymer, which is cross-recognized by the CNS but is not encephalitogenic. The effect of Cop-1 here is reminiscent of the effect obtained with a cryptic epitope of MBP (6). We suggest that the T cell immune response to the polymer, obtained either by adoptive transfer or immunization at the time of the injury, seems to provide an effective means of posttraumatic protection. It remains to be seen whether the activity of Cop-1 as a neuroprotectant rather than as a suppressor depends on the way of its administration. It is also essential to find out how the locally accumulated CNS-specific T cells or T cells specific to cross-reactive antigens such as Cop-1, mediate neuroprotection in the context of CNS injuries. The T cell-mediated neuroprotection demonstrated here might be applicable to both chronic and acute injuries of CNS nerves, in which neurons are vulnerable to degeneration and amenable to neuroprotection (7, 34).

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- Jonas, J. B. & Budde, W. M. (2000) *Prog. Retin. Eye Res.* **19**, 1–40.
- Steinsapir, K. D. (1999) *Curr. Opin. Ophthalmol.* **10**, 340–342.
- Schwartz, M., Belkin, M., Yoles, E. & Solomon, A. (1996) *J. Glaucoma* **5**, 427–432.
- Yoles, E. & Schwartz, M. (1998) *Surv. Ophthalmol.* **42**, 367–372.
- Moalem, G., Yoles, E., Leibowitz-Amit, R., Muller-Gilior, S., Mor, F., Cohen, I. R. & Schwartz, M. (2000) *J. Neuroimmunol.*, in press.
- Moalem, G., Leibowitz-Amit, R., Yoles, E., Mor, F., Cohen, I. R. & Schwartz, M. (1999) *Nat. Med.* **5**, 49–55.
- Schwartz, M. & Yoles, E. (2000) *Curr. Opin. Ophthalmol.* **11**, 107–111.
- Hauben, E., Nevo, U., Yoles, E., Moalem, G., Agranov, E., Mor, F., Akselrod, S., Neeman, M., Cohen, I. R. & Schwartz, M. (2000) *Lancet* **355**, 286–287.
- Teitelbaum, D., Meshorer, A., Hirshfeld, T., Arnon, R. & Sela, M. (1971) *Eur. J. Immunol.* **1**, 242–248.
- Teitelbaum, D., Fridkis-Hareli, M., Arnon, R. & Sela, M. (1996) *J. Neuroimmunol.* **64**, 209–217.
- Teitelbaum, D., Sela, M. & Arnon, R. (1997) *Isr. J. Med. Sci.* **33**, 280–284.
- Ben-Nun, A., Wekerle, H. & Cohen, I. R. (1981) *Eur. J. Immunol.* **11**, 195–199.
- Gillis, S., Ferm, M. M., Ou, W. & Smith, K. A. (1978) *J. Immunol.* **120**, 2027–2032.
- Ben-Nun, A. & Cohen, I. R. (1982) *J. Immunol.* **129**, 303–308.
- Duvdevani, R., Rosner, M., Belkin, M., Sautter, J., Sabel, B. A. & Schwartz, M. (1990) *Restor. Neurol. Neurosci.* **2**, 31–38.
- Yoles, E. & Schwartz, M. (1998) *Exp. Neurol.* **153**, 1–7.
- Hunig, T., Wallny, H. J., Hartley, J. K., Lawetzky, A. & Tiefenthaler, G. (1989) *J. Exp. Med.* **169**, 73–86.
- Hirschberg, D. L., Moalem, G., He, J., Mor, F., Cohen, I. R. & Schwartz, M. (1998) *J. Neuroimmunol.* **89**, 88–96.
- Moalem, G., Monsonago, A., Shani, Y., Cohen, I. R. & Schwartz, M. (1999) *FASEB J.* **13**, 1207–1217.
- Kerschensteiner, M., Gallmeier, E., Behrens, L., Leal, V. V., Misgeld, T., Klinkert, W. E., Kolbeck, R., Hoppe, E., Oropeza-Wekerle, R. L., Bartke, I., et al. (1999) *J. Exp. Med.* **189**, 865–870.
- Weiner, H. L. (1997) *Immunol. Today* **18**, 335–343.
- Weiner, H. L. (1997) *Annu. Rev. Med.* **48**, 341–351.
- Ben-Nun, A., Mendel, I., Bakimer, R., Fridkis-Hareli, M., Teitelbaum, D., Arnon, R., Sela, M. & Kerlero de Rosbo, N. (1996) *J. Neurol.* **243**, S14–S22.
- Webb, C., Teitelbaum, D., Arnon, R. & Sela, M. (1973) *Immunol. Commun.* **2**, 185–192.
- Teitelbaum, D., Aharoni, R., Arnon, R. & Sela, M. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 9724–9728.
- Aharoni, R., Teitelbaum, D., Sela, M. & Arnon, R. (1998) *J. Neuroimmunol.* **91**, 135–146.
- Fridkis-Hareli, M., Aharoni, R., Teitelbaum, D., Arnon, R., Sela, M. & Strominger, J. L. (1999) *Int. Immunol.* **11**, 635–641.
- Aharoni, R., Teitelbaum, D. & Arnon, R. (1993) *Eur. J. Immunol.* **23**, 17–25.
- Aharoni, R., Teitelbaum, D., Sela, M. & Arnon, R. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 10821–10826.
- Hickey, W. F., Hsu, B. L. & Kimura, H. (1991) *J. Neurosci. Res.* **28**, 254–260.
- Yan, Q., Elliott, J. & Snider, W. D. (1992) *Nature (London)* **360**, 753–755.
- Sendtner, M., Holtmann, B., Kolbeck, R., Thoenen, H. & Barde, Y. A. (1992) *Nature (London)* **360**, 757–759.
- Schwartz, M., Moalem, G., Leibowitz-Amit, R. & Cohen, I. R. (1999) *Trends Neurosci.* **22**, 295–299.
- Schwartz, M. & Yoles, E. (2000) *Invest. Ophthalmol. Visual Sci.* **41**, 349–351.

Differential T cell response in central and peripheral nerve injury: connection with immune privilege

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ABSTRACT The central nervous system (CNS), unlike the peripheral nervous system (PNS), is an immune-privileged site in which local immune responses are restricted. Whereas immune privilege in the intact CNS has been studied intensively, little is known about its effects after trauma. In this study, we examined the influence of CNS immune privilege on T cell response to central nerve injury. Immunocytochemistry revealed a significantly greater accumulation of endogenous T cells in the injured rat sciatic nerve than in the injured rat optic nerve (representing PNS and CNS white matter trauma, respectively). Use of the *in situ* terminal deoxynucleotidyl transferase-catalyzed DNA nick end labeling (TUNEL) procedure revealed extensive death of accumulating T cells in injured CNS nerves as well as in CNS nerves of rats with acute experimental autoimmune encephalomyelitis, but not in injured PNS nerves. Although Fas ligand (FasL) protein was expressed in white matter tissue of both systems, it was more pronounced in the CNS. Expression of major histocompatibility complex (MHC) class II antigens was found to be constitutive in the PNS, but in the CNS was induced only after injury. Our findings suggest that the T cell response to central nerve injury is restricted by the reduced expression of MHC class II antigens, the pronounced FasL expression, and the elimination of infiltrating lymphocytes through cell death.—Moalem, G., Monsonego, A., Shani, Y., Cohen, I. R., Schwartz, M. Differential T cell response in central and peripheral nerve injury: connection with immune privilege. *FASEB J.* 13, 1207–1217 (1999)

Key Words: T lymphocytes • CNS • PNS • Fas ligand • major histocompatibility complex

MAINTENANCE OF NEURAL functions is vital for vertebrate survival, and loss of these functions as a result of local immune responses may threaten central nervous system (CNS)² integrity. To maintain proper neural functioning, the CNS may have had to evolve mechanisms that limit its vulnerability to irreversible modifications that might be caused by immune reactions. The CNS, in contrast to the peripheral

nervous system (PNS), is an immune-privileged site (1, 2). The concept of CNS immune privilege is supported by the prolonged survival of allografts within the brain parenchyma (3, 4), the presence of a blood-brain barrier (5), the absence of typical lymphatic drainage (6), the reduced expression of major histocompatibility complex (MHC) class I and II antigens (7, 8), unconventional antigen-presenting cells (9), a low concentration of complement components (10), and immunosuppression by the CNS microenvironment (11, 12). Immune privilege in the CNS was long thought to be maintained by 'immune ignorance' (13). It was believed that the CNS is isolated from the immune system, preventing antigen escape and excluding immune cells, and that consequently the immune system simply ignores the area. Over the years it has become clear that antigens from the CNS can escape and induce immune responses in the periphery (10, 14), and that the CNS is accessible to activated antigen-specific lymphocytes (15). Since antigens originating in the CNS can reach and influence the systemic immune apparatus and because immune cells can gain access to the CNS, immune ignorance is no longer a valid explanation of CNS privilege.

Studies of CNS inflammation caused by autoimmune myelin-specific T cells in different states of activation revealed that the blood-brain barrier effectively prevents resting T cells from entering the CNS parenchyma. These studies demonstrated, however, that activated myelin-specific T cells are able to pass

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² Abbreviations: BSA, bovine serum albumin; CNP, cyclic nucleotide phosphohydrolase; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; FasL, Fas ligand; FCS, fetal calf serum; GFAP, glial fibrillary acidic protein; IFN, interferon; IgG, immunoglobulin G; IL, interleukin; i.p., intraperitoneal; MBP, myelin basic protein; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PDL, poly-D-lysine; PMSF, phenylmethylsulfonylfluoride; PNS, peripheral nervous system; RPL19, ribosomal protein L19; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCR, T cell receptor; TUNEL, terminal deoxynucleotidyl transferase-catalyzed DNA nick end labeling.

through the blood-brain barrier and initiate CNS lesions causing autoimmune disease (16), thus suggesting that access to the CNS is restricted to T cells that are activated (9). It was further shown that activated T cells, irrespective of their antigen specificity, enter the CNS parenchyma within hours of injection, but only cells capable of reacting with a CNS antigen can persist there (15).

After CNS injury, the recruitment of macrophages is delayed and limited compared with the strong macrophage response after PNS injury (17). CNS injury may also be accompanied by the infiltration of T lymphocytes into the site of the lesion (18). Although such infiltration might imply the possibility of a classic immune response within the damaged CNS, the effect of the immune-privileged status on these infiltrating T cells is not yet known. In the present study, we examined whether the dialog between T cells and the CNS differs from the T cell-PNS dialog in response to injury. Using partial crush injuries of the optic and sciatic nerves as models for CNS and PNS white matter trauma, respectively, we demonstrate differential T cell response to injury of the central and peripheral nerves. In the injured sciatic nerve, T cell accumulation is significantly greater than in the injured optic nerve. Elimination of T cells through cell death occurs extensively in the optic nerve after injury and in rats with experimental autoimmune encephalomyelitis (EAE), but only to a very small extent in the injured sciatic nerve. Moreover, MHC class II antigens are constitutively expressed in the sciatic nerve, but are induced only after injury in the optic nerve. Fas ligand (FasL) mRNA is expressed in both optic and sciatic nerves, but FasL protein is more pronounced in the optic nerve. These results suggest that the immune-privileged CNS restricts the T cell response to nerve injury.

MATERIALS AND METHODS

Animals

Inbred female Lewis rats (8–12 wk old) and newborn Wistar or Lewis rats were supplied by the Animal Breeding Center of the Weizmann Institute of Science. The rats were housed in a light- and temperature-controlled room and matched for age and sex in each experiment. Animals were used according to the regulations formulated by the Institutional Animal Care and Use Committee.

T cells

A T cell line specific for myelin basic protein (MBP) was generated from draining lymph node cells obtained from Lewis rats immunized with MBP antigen, which was prepared from guinea pig spinal cord as described previously (19). MBP was dissolved in 1 mg/ml of phosphate-buffered saline (PBS) and emulsified with an equal volume of Freund's

incomplete adjuvant (Difco Laboratories, Detroit, Mich.) supplemented with 4 mg/ml of *Mycobacterium tuberculosis* (Difco). Ten days after the antigen was injected into their hind foot pads in 0.1 ml of the emulsion, the rats were killed and their draining lymph nodes were surgically removed and dissociated. The cells were washed and activated with MBP antigen (10 µg/ml) in proliferation medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), nonessential amino acids (1 ml/100 ml), and autologous rat serum 1% (v/v). After incubation for 72 h at 37°C, 90% relative humidity, and 7% CO₂, the cells were transferred to propagation medium consisting of DMEM, L-glutamine, 2-mercaptoethanol, sodium pyruvate, nonessential amino acids, and antibiotics in the same concentrations as above, with the addition of 10% fetal calf serum (FCS) (v/v) and 10% T cell growth factor derived from the supernatant of concanavalin A-stimulated spleen cells. Cells were grown in propagation medium for 4–10 days before being restimulated with their antigen (10 µg/ml) in the presence of irradiated (2000 rad) thymus cells (10^7 cells/ml) in proliferation medium. The T cell line was expanded by repeated stimulation and propagation (20).

Glial cells

Primary cultures of glial cells were prepared by a modification of the procedure of McCarthy and de Vellis (21). Cells dissociated from the cerebral cortex of 2-day-old rats were cultured in poly-D-lysine (PDL)-coated tissue culture flasks (2 brains/85 cm² flask) containing DMEM, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% FCS. The medium was changed after 24 h and every 2 days thereafter. To obtain pure cultures of microglia, after 8 days the flasks were shaken at 37°C on a rotary platform for 6 h and the detached cells were collected and seeded on PDL-coated coverslips in 24-well plates (10^5 cells/ml in each well) in RPMI 1640 medium supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 10% FCS. Fresh medium was added to the flasks, which were then shaken for an additional 18 h at 37°C. The detached cells, consisting mostly of progenitor cells for oligodendrocytes and type 2 astrocytes, were collected and seeded on PDL-coated coverslips in 24-well plates (5×10^4 cells/ml in each well). To encourage oligodendrocyte development, seeding was carried out in Raff's modification of Bottenstein and Sato's defined medium (22, 23). Fresh medium was again added to the flasks, and 50 µl of 25 mM cytosine-β-D-arabinofuranoside (Sigma, St. Louis, Mo.) was added 1 day later. After 24 h, the medium was replaced by a defined medium for astrocytes consisting of DMEM, 2 mM glutamine, 0.1 mg/ml transferrin, 0.1% free fatty acid bovine serum albumin (BSA), 0.1 mM putrescine, 0.45 mM L-thyroxine, and 0.224 mM sodium selenite. The astrocytes were trypsinized and plated on PDL-coated coverslips in 24-well plates (5×10^4 cells/ml in each well).

Crush injury of optic and sciatic nerves

Crush injury of the optic nerve was performed as described previously (24, 25). Briefly, rats were deeply anesthetized by intraperitoneal (i.p.) injection of Rompun (xylazine, 10 mg/kg; Vitamed, Bat-Yam, Israel) and Vetalar (ketamine, 50 mg/kg; Fort Dodge Laboratories, Fort Dodge, Iowa). Using a binocular operating microscope, a lateral canthotomy was performed in the right eye and the conjunctiva was incised lateral to the cornea. After separation of the refractor bulbi

muscles, the optic nerve was exposed intraorbitally by blunt dissection. Using calibrated cross-action forceps, the optic nerve was subjected to a crush injury 2 mm from the eye. The uninjured contralateral nerve was left undisturbed. The sciatic nerve was crushed under deep anesthesia, as described previously (26). The sciatic nerve was exposed and a similar crush injury was inflicted, after which the skin was sutured.

Immunocytochemistry

Longitudinal cryosections (20 μ m thick) of the nerves were picked up onto gelatin-coated glass slides and frozen until preparation for fluorescence staining. The sections were fixed in ethanol for 10 min at room temperature, washed twice with double-distilled water, and incubated for 3 min in PBS containing 0.05% polyoxyethylene-sorbitan monolaurate (Tween-20). For immunostaining of the cells, coverslips were fixed in methanol for 15 min at -20°C , washed three times with PBS, fixed in acetone for 2 min at room temperature, and again washed three times with PBS. Sections or cells were then incubated for 1 h at room temperature with mouse anti-rat monoclonal antibodies to T cell receptor (TCR) (27), glial fibrillary acidic protein (GFAP) (BioMakor, Rehovot, Israel), FasL (Transduction Laboratories, Lexington, Ky.), ED1 (Serotek, Oxford, U.K.), MHC class II antigens (OX-6) (Serotek, Oxford, U.K.), and cyclic nucleotide phosphohydrolase (CNP) (Promega, Madison, Wis.) or rabbit anti-rat FasL polyclonal antibody (Santa Cruz, Calif.), diluted in PBS containing 3% FCS and 2% BSA. The sections or cells were then washed three times with PBS containing 0.05% Tween-20 and incubated with fluorescein isothiocyanate (FITC)- or Cy3-conjugated goat anti-mouse immunoglobulin G (IgG) (with minimal cross-reaction to rat, human, bovine and horse serum proteins; Jackson ImmunoResearch, West Grove, Pa.) or rhodamine (TRITC)-conjugated goat anti-rabbit IgG (Jackson), for 1 h at room temperature. The sections or cells were washed with PBS containing Tween-20 and treated with glycerol containing 1,4-diazobicyclo-(2, 2, 2) octane to inhibit quenching of fluorescence. The sections and cells were viewed with a Zeiss Universal fluorescence microscope using filters that detect either FITC or Cy3 and TRITC.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from rat optic and sciatic nerves 7 days after injury, as well as from the uninjured nerves and from spleen (using the TRI reagent; Molecular Research Center, Cincinnati, Ohio), according to the manufacturer's instructions. From each sample, 1 μ g of total RNA was reverse-transcribed to cDNA using a thermal program of 42°C for 60 min and 95°C for 2 min. Aliquots from each cDNA preparation were amplified by PCR, using the following primers specific for rat FasL and for rat ribosomal protein L19 (RPL19): FasL (forward, 5'-GTTTTTCTTGTCATCCTC-3'; reverse, 5'-GCCGCCCTTCTTATACTTC-3') and RPL19 (forward, 5'-CTGAAGGTCAAAGGGAATGTG-3'; reverse, 5'-GGACAGATCTTGATGATCTC-3'), giving a 447 bp and a 194 bp product, respectively. The PCR conditions for rat FasL were 30 s for denaturation at 94°C , 1 min of annealing at 60°C , and 2 min of elongation at 72°C for 35 cycles. The PCR conditions for rat RPL19 were 30 s for denaturation at 94°C , 1 min of annealing at 60°C , and 2 min of elongation at 72°C for 25 cycles. The products were resolved on a 1% agarose gel. The FasL PCR fragment was then isolated from the gel using GenElute agarose spin columns (Supelco, Bellefonte, Pa.) and sequenced.

Immunoblot (Western blot) analysis

Glial cells were extracted with a lysis buffer containing Tris (10 mM, pH 7.5), NaCl (150 mM), Triton X-100 (1%), EDTA (1 mM), spermidine (1 mM), aprotinin (25 μ g/ml), leupeptin (25 μ g/ml), pepstatin (5 μ g/ml), and phenylmethylsulfonylfluoride (PMSF) (1 mM) for 2 h at 4°C with gentle shaking, and the supernatant was collected. For preparation of a high-speed supernatant derived from optic or sciatic nerves, nerves were removed by dissection, frozen in liquid nitrogen, and homogenized immediately in a lysis buffer containing Tris acetate (50 mM), pepstatin (5 μ g/ml), leupeptin (25 μ g/ml), aprotinin (5 μ g/ml), and PMSF (1 mM). The supernatants were collected after high-speed centrifugation (110,000 g). Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel), followed by blotting onto a nitrocellulose membrane for 2 h at 200 mA (in Tris-glycine). The membrane was incubated overnight at 4°C with PBS containing 5% (v/v) skim milk, incubated with monoclonal antibody to FasL (Transduction Laboratories) in PBS containing 5% skim milk for 1.5 h at room temperature, and washed three times for 20 min in PBS containing 0.05% Tween-20. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) in PBS containing 5% skim milk for 1.5 h at room temperature and washed three times for 20 min in PBS containing 0.05% Tween-20. Immunoreactive bands were visualized by the enhanced chemiluminescence method (ECL, Amersham, U.K.).

In situ detection of cell death by terminal deoxynucleotidyl transferase-catalyzed DNA nick end labeling (TUNEL)

Seven days after crush injury of the optic or sciatic nerves, the rats were killed and their nerves were removed and processed for cryosectioning. Frozen sections were fixed in a 10% formalin solution for 10 min at room temperature and washed twice for 5 min in PBS. The sections were then transferred to 100% methanol for 15 min at -20°C , and washed twice for 5 min with PBS. The samples were rehydrated by serial washings for 5 min in ethanol 100%, 95%, and 70%, and then incubated for 10 min with PBS. For permeabilization, proteases were digested with proteinase K for 20 min at room temperature. Labeling of the ends of the DNA fragments was performed using an *in situ* apoptosis detection kit (Genzyme, Cambridge, Mass.) according to the manufacturer's instructions. The labeled ends were detected using the fluorescein detection kit supplied with a streptavidin-fluorescein conjugate. The fluorescein-stained cells were visualized using a fluorescence microscope.

Analysis of cell numbers in nerve sections

Immunostained cells or TUNEL-reactive cells in each nerve section were counted at the site of injury (discerned by morphology) and at randomly selected areas in the uninjured nerves, using the fluorescence microscope. Each group contained three or four rats. For each nerve, two to four sections were counted and the numbers per mm^2 were calculated and averaged. The results were analyzed using the InStat program. Data were analyzed using one-way analysis of variance, Bartlett's test for homogeneity of variances, and a subsequent Bonferroni multiple comparison *t* test. To detect double-labeled T cells and TUNEL-reactive cells, sections were stained for TUNEL and then immunostained with anti-TCR antibody. Because of high background in the immunostained sections induced by the TUNEL procedure, adjacent sections were also stained for TUNEL or T cells, photographed,

scanned to the computer, analyzed by overlapping images, and documented.

RESULTS

Endogenous T cell accumulation is significantly more pronounced in the injured PNS than in the injured CNS

To compare T cell accumulation at sites of CNS and PNS axonal crush injury, optic and sciatic nerves were excised from rats 3, 7, 14, or 21 days after injury, cryosectioned, and analyzed immunohistochemically for the presence of T cells, using an antibody that recognizes T cells exclusively. Measurement of the numbers of immunolabeled T cells at the injury site revealed a significantly greater accumulation of T cells in the injured sciatic nerve than in the injured optic nerve (Fig. 1). In the PNS, large numbers of T cells were detected at the site of the injury and distal to it by day 3. The numbers of T cells reached a peak on day 7 and decreased thereafter. In the CNS, however, T cells arrived in fewer numbers and were localized to a more restricted area

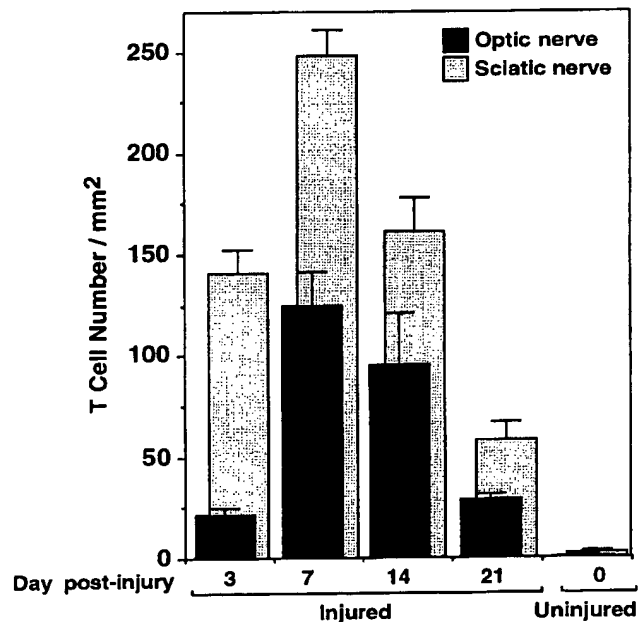


Figure 1. Greater accumulation of endogenous T cells in injured sciatic nerve than in injured optic nerve. At 3, 7, 14, or 21 days after optic or sciatic nerve injury, the injured nerves and the contralateral uninjured nerves were removed, cryosectioned, and analyzed immunohistochemically for the presence of immunolabeled T cells. The histogram shows the mean numbers of T cells \pm SE. Statistical analysis (ANOVA) reveals significant differences in T cell numbers between injured optic nerve and injured sciatic nerve on day 3 ($P < 0.001$), day 7 ($P < 0.001$), and day 14 ($P < 0.05$), but not on day 21 ($P > 0.05$).

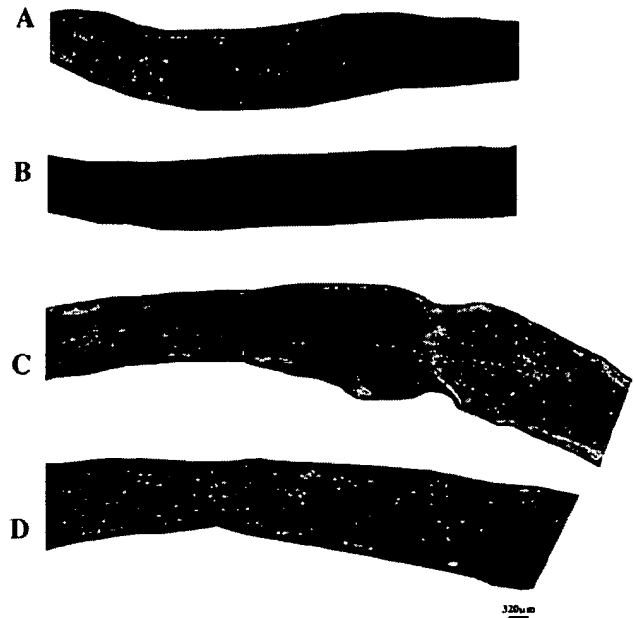


Figure 2. Expression of MHC class II antigens in optic and sciatic nerves. Cryosections taken from injured optic nerve (A), uninjured optic nerve (B), injured sciatic nerve (C), and uninjured sciatic nerve (D) were immunostained for MHC class II antigens using OX-6 antibody detected with FITC antibody to mouse IgG. Note the absence of staining in uninjured optic nerve, unlike in uninjured sciatic nerve. After injury, however, staining was detected in the optic nerve and was increased in the sciatic nerve.

around the injury site. As in the PNS, the peak occurred on day 7, after which their numbers decreased.

MHC class II antigens are constitutively expressed in PNS white matter, but are induced only after injury in CNS white matter

MHC class II molecules on antigen-presenting cells play a key role in presentation of antigens to T cells. We examined uninjured and injured optic and sciatic nerves for MHC class II expression 7 days after crush injury by immunostaining with the OX-6 antibody. MHC class II (Ia) antigens were expressed in the uninjured sciatic nerve, but not in the uninjured optic nerve (Fig. 2). Positive immunostaining was observed on scattered interstitial cells. After injury, expression of MHC class II antigens was increased in the sciatic nerve and induced in the optic nerve. Nevertheless, expression of MHC class II antigens in the injured optic nerve was much lower than in the injured sciatic nerve. These results indicate that the white matter of rat PNS constitutively expresses MHC class II antigens, in contrast to rat CNS white matter, where the expression of MHC class II antigens is induced only after injury and is less pronounced.

Death of infiltrating T cells in the CNS 1 wk after injury

To examine the possibility that infiltrating T cells die in the injured CNS, we performed the *in situ* TUNEL procedure, followed by immunohistochemical analysis using anti-TCR antibody. Although TUNEL is used to measure apoptotic cell death, it is reasonable to assume that it might detect necrotic cell death as well, since DNA degradation is an inevitable, albeit late, event in this process. Optic and sciatic nerves were examined 1 wk after crush injury. Extensive T cell infiltration without associated death occurred in the injured sciatic nerve (Fig. 3). In contrast, a high level of death among the infiltrating T cells was

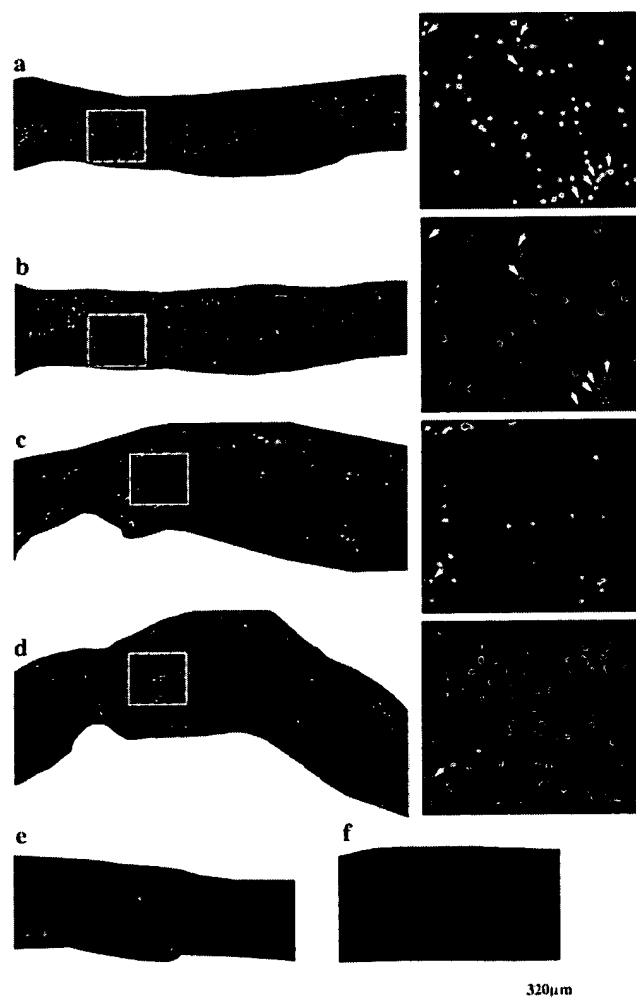


Figure 3. *In situ* detection of TUNEL-reactive cells and immunostained T cells in optic and sciatic nerves. Cryosections from the same injured optic nerve tissue obtained 1 wk after injury were stained for (a) TUNEL-reactive cells and (b) T cells. Cryosections from the same injured sciatic nerve tissue obtained 1 wk after injury were stained for (c) TUNEL-reactive cells, and (d) T cells. The arrowheads indicate cells that were double-labeled by TUNEL staining and TCR immunocytochemistry. Cryosections of (e) uninjured optic nerve and (f) uninjured sciatic nerve stained for TUNEL-reactive cells.

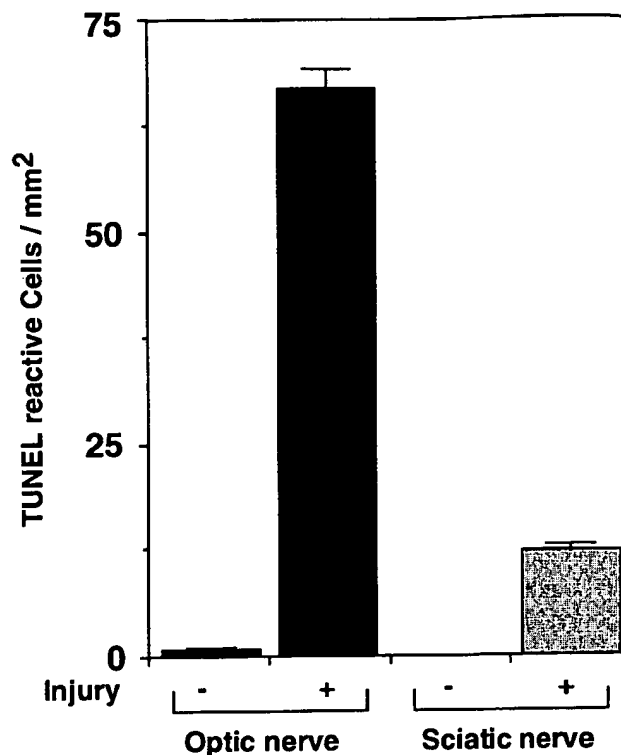


Figure 4. Quantification of TUNEL-reactive cells in injured optic and sciatic nerves. Cryosections were analyzed by TUNEL staining 1 wk after injury. The histogram shows the mean number of TUNEL-reactive cells \pm SE. Statistical analysis (ANOVA) revealed significant difference in numbers of TUNEL-reactive cells between injured optic nerve and injured sciatic nerve ($P < 0.001$).

observed in the injured optic nerve. About 20% of the T cells in the optic nerve and less than 2% in the sciatic nerve were identified as TUNEL reactive. The numbers of TUNEL-reactive cells observed in injured and uninjured optic and sciatic nerves are shown in Fig. 4. Whereas more T cells were observed in the sciatic nerve than in the optic nerve after injury, the numbers of TUNEL-reactive cells were higher in the optic nerve. These results suggest that cell death constitutes at least part of the mechanism regulating T cell elimination in the injured CNS.

T cell accumulation in injured and uninjured CNS is increased after injection of anti-MBP T cells and is accompanied by cell death

To learn whether T cell elimination also occurs in the CNS of rats with EAE, we examined T cell accumulation, disappearance, and death in crush-injured and uninjured optic nerve tissues of Lewis rats injected with anti-MBP T cells. The T cell line used for this experiment (T_{MBP}) can home to and affect the white matter of the CNS (16). The T cell line was activated with MBP for 3 days and then injected i.p. (10×10^6 cells) into rats a few minutes

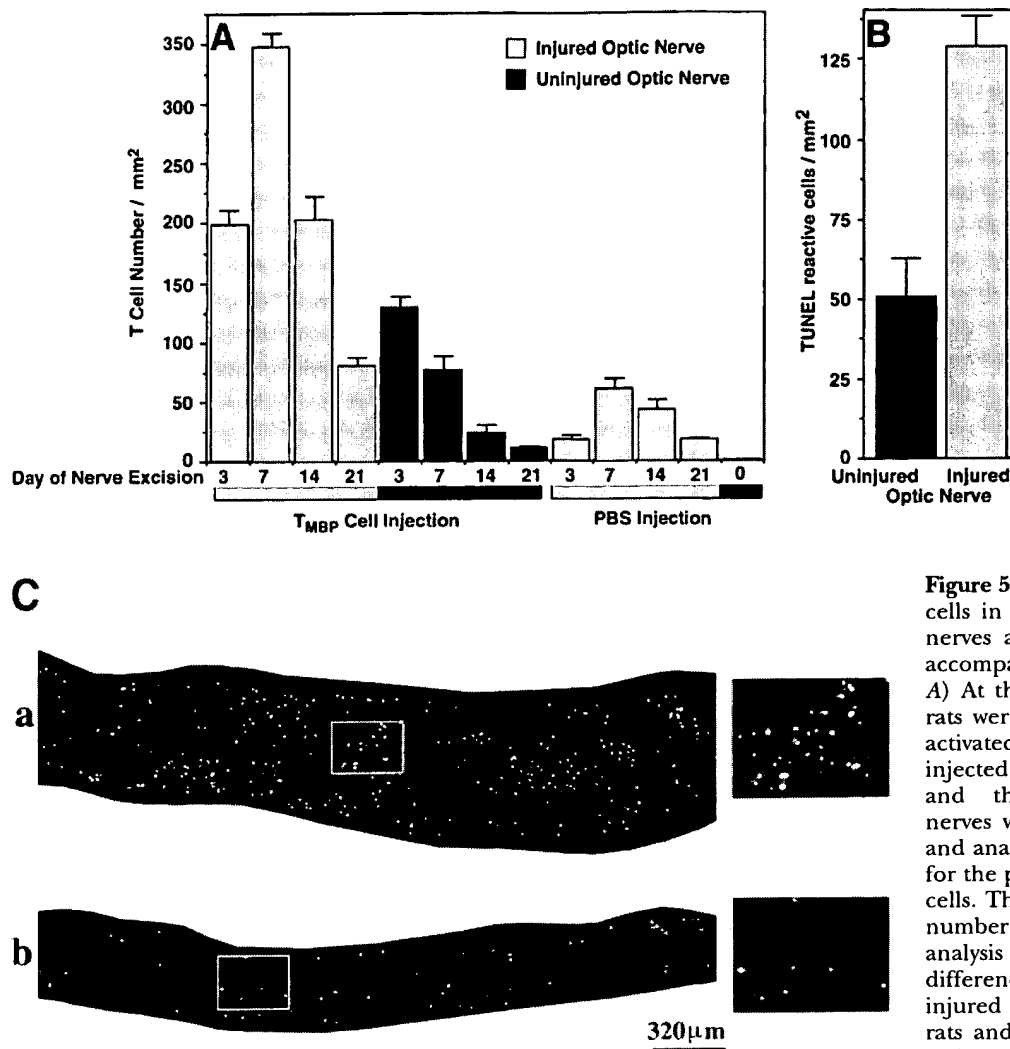


Figure 5. Increased accumulation of T cells in injured and uninjured optic nerves after injection of T_{MBP} cells, accompanied by extensive cell death. **A)** At the time of optic nerve crush, rats were injected i.p. with 10×10^6 activated T_{MBP} cells. Control rats were injected i.p. with PBS. Injured nerves and the contralateral uninjured nerves were removed, cryosectioned, and analyzed immunohistochemically for the presence of immunolabeled T cells. The histogram shows the mean number of T cells \pm SE. Statistical analysis (ANOVA) revealed significant differences in T cell numbers between injured optic nerve of T_{MBP}-injected rats and injured optic nerve of control rats on day 3 ($P < 0.001$), day 7

($P < 0.001$), day 14 ($P < 0.001$), and day 21 ($P < 0.01$), and between injured and uninjured optic nerves of T_{MBP}-injected rats on days 3, 7, 14, and 21 ($P < 0.001$). **B)** Cell death was assessed by TUNEL staining 1 wk after T_{MBP} cell injection. The histogram shows the mean numbers of TUNEL-reactive cells \pm SE. Statistical analysis (ANOVA) revealed significant difference in numbers of TUNEL-reactive cells between injured and uninjured optic nerve ($P < 0.001$). **C)** Photomicrographs of (a) injured and (b) uninjured optic nerves of T_{MBP}-injected rats stained for TUNEL.

after injury. The injected rats developed EAE within 3–4 days. Control rats were injected i.p. with PBS. At 3, 7, 14, or 21 days after injury, both the injured and the uninjured optic nerves were excised, cryosectioned, and analyzed immunohistochemically for the presence of immunolabeled T cells. Cell death was assessed by TUNEL in injured and uninjured optic nerves at the peak of T cell accumulation. T cells were detected in the injured optic nerve by day 3, increased to a peak on day 7, and then decreased in number by day 21 (Fig. 5A). At all time points examined, the numbers of T cells detected in the injured optic nerves of T_{MBP}-injected rats were significantly greater than in the injured optic nerves of PBS-injected rats. Fewer T cells were seen in the uninjured nerves of T_{MBP}-injected rats, and their numbers decreased from day 3 to day 21 after the injection. No T cells were detected in uninjured

optic nerves of PBS-injected control rats. Although TUNEL-reactive cells were detected in the uninjured optic nerves, their numbers were significantly greater in the injured optic nerves 1 wk after T_{MBP} cell injection (Fig. 5B, C), in correlation with the T cell numbers. In both injured and uninjured optic nerves of T_{MBP}-injected rats, ~30% of the T cells were identified as TUNEL reactive (data not shown). During the same period, the number of T cells declined and the animals recovered from the disease. These results suggest that the CNS ability to eliminate T cells does not depend on injury.

Expression of FasL in the white matter of the rat nervous system

Because some tissues appear to require FasL in order to exhibit immune-privileged status by killing infil-

trating lymphocytes and inflammatory cells (28, 29), we examined FasL expression in the rat nervous system. RT-PCR analysis of total RNA isolated from crush-injured and uninjured optic nerves and sciatic nerves showed that FasL mRNA is expressed in both the CNS and the PNS white matter, whether injured or not (Fig. 6). The PCR product was sequenced and was found to be homologous to rat FasL.

To examine the expression of FasL protein in the CNS and PNS white matter, we performed immunohistochemical analyses of uninjured sciatic nerves as well as of injured and uninjured optic nerves, using anti-FasL antibody. At the same time, we used anti-ED1 antibody to detect reactive microglia and anti-GFAP antibody to detect astrocytes in the injured optic nerve (Fig. 7). Intensive FasL staining, corresponding to the ED1-immunoreactive microglia, was detected at the injury site. Staining was weak in the uninjured optic nerve, and even weaker in the uninjured sciatic nerve (Fig. 7). Different patterns and intensities of FasL staining were observed in the injured and uninjured optic nerves, suggesting differential states of regulation and possibly a different physiological role for FasL under normal conditions compared with trauma.

In an attempt to ascribe FasL immunoreactivity to a particular cell type within the optic nerve as a

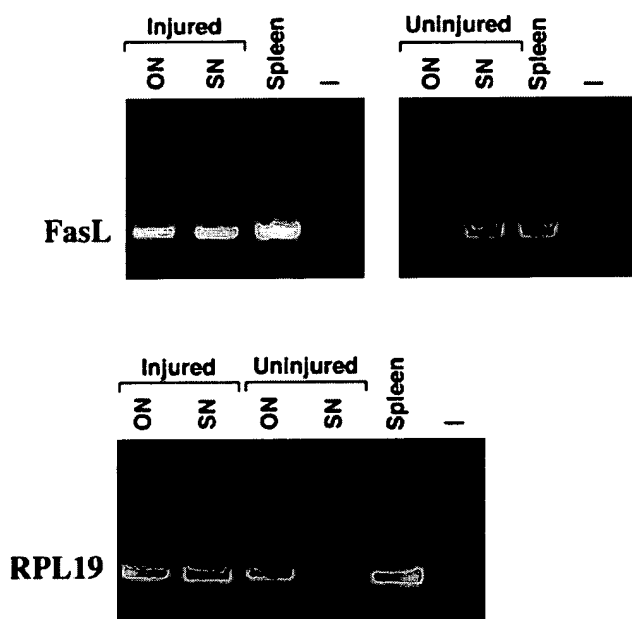


Figure 6. FasL mRNA expression in rat optic and sciatic nerves determined by RT-PCR. RT-PCR analysis was performed on RNA isolated from injured optic nerve (ON), injured sciatic nerve (SN), uninjured optic nerve, uninjured sciatic nerve, and spleen (positive control); each sample was analyzed without the reverse transcriptase enzyme (—) to exclude the possibility of DNA contamination (negative control). FasL was expressed in both rat optic and sciatic nerves, as well as in the spleen. RPL19 was used as a control for the RT-PCR analysis of the isolated RNA.

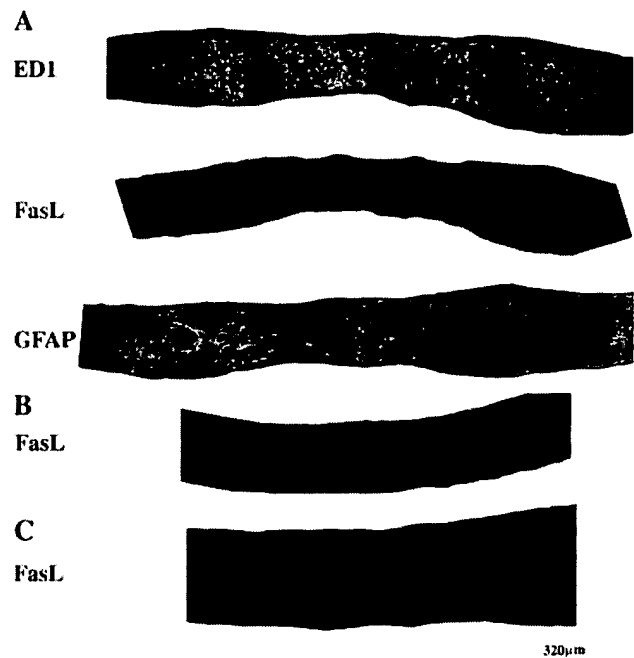


Figure 7. Expression of FasL by optic and sciatic nerves. Cryosections taken from (A) injured optic nerve 1 wk after injury, (B) uninjured optic nerve, and (C) uninjured sciatic nerve were immunostained for FasL using a monoclonal antibody detected with a Cy3 antibody. The same injured optic nerve tissue (A) was stained with anti-ED1 antibody to detect activated microglia and macrophages and with anti-GFAP antibody to visualize the injury site (lack of GFAP-positive astrocytes) detected with a FITC antibody. Staining of each of the tissues in the absence of the first antibody was negative.

possible mediator of T cell apoptosis, we analyzed primary cultures of astrocytes, microglia, and oligodendrocytes using anti-FasL antibody and double staining with a specific marker for each cell population. The results pointed to the constitutive expression of FasL in the primary cultures of CNS glial cells (Fig. 8). Similar results were obtained by Western blot analysis (Fig. 9). Glial cell lysates (astrocytes, microglia, and oligodendrocytes) (Fig. 9A) and high-speed supernatants of extracts obtained from injured and uninjured optic nerves (Fig. 9B) exhibited intense immunoreactive bands recognized by monoclonal anti-FasL antibody. Weaker bands were detected in high-speed supernatants of uninjured and injured sciatic nerves (Fig. 9B). The 40 kDa protein observed in the samples seems to be FasL expressed on the cell membrane. The identity of the upper band at M_r 80 kDa is not known. It might correspond to a soluble form of rat FasL (sFasL), as reported for human FasL (30).

Taken together, these findings confirm the expression of FasL in the white matter of the rat nervous system and demonstrate that all CNS glial cells are capable of expressing FasL protein.

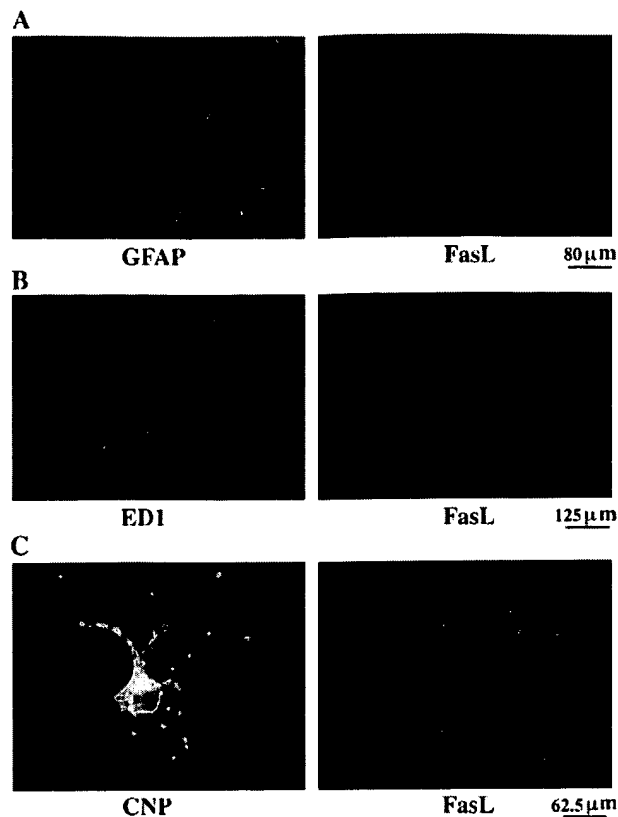


Figure 8. Localization of FasL protein in primary cultures of CNS glial cells by immunohistochemical analysis using polyclonal anti-FasL antibody. Double immunostaining was performed on (A) primary cultures of astrocytes using mouse anti-rat GFAP and rabbit anti-rat FasL, (B) primary microglial cultures using mouse anti-rat ED1 and rabbit anti-rat FasL, and (C) primary oligodendrocyte cultures using mouse anti-rat CNP and rabbit anti-rat FasL. The mouse antibodies were detected using FITC goat anti-mouse IgG and the rabbit antibody was detected using TRITC goat anti-rabbit IgG. In each case, staining of the cells with any of the first mouse antibodies, followed by the second anti-rabbit IgG, or staining with the first rabbit antibody, followed by the second anti-mouse IgG, or staining with the second antibody only, was negative.

DISCUSSION

This study demonstrates that the T cell response to injury in the CNS differs fundamentally from that in the PNS. It has already been established that the CNS, unlike the PNS, is an immune-privileged site (1, 2) and that the immune-privileged status has profound implications for macrophage recruitment and activation after axonal injury (31–33). The mechanism responsible for immune privilege in the CNS is not fully understood, and even less is known about the effects of immune privilege under traumatic conditions. We show that the accumulation of endogenous T cells is significantly greater after PNS trauma than after CNS trauma. Moreover, in contrast to the extensive death of infiltrating T cells in the injured CNS and in rats with EAE, hardly any cell death was detectable in the T cells accumulating in

the injured PNS. We further show that MHC class II antigens are expressed by the intact PNS but not by the intact CNS, though their expression in the CNS can be induced by trauma. Finally, we show that FasL is expressed in both CNS and PNS white matter, but more strongly in the CNS.

These findings suggest that T cell-associated immune reactions occur in the CNS white matter after injury and in cases of autoimmune disease. Nevertheless, the T cells appear to be gradually eliminated. Injuries to axons in both the CNS and PNS of mammals result in axonal degeneration distal to the site of the lesion (Wallerian degeneration). However, in contrast to the PNS, axons in the CNS do not regenerate (17). In the present study, a similar course of T cell accumulation was observed after crush injury in the nonregenerative optic nerve and in the regenerative sciatic nerve. In the PNS a few days after injury, large numbers of T cells were seen throughout the nerve, but they disappeared, in temporal correlation with nerve regrowth. In the CNS, however, T cells were recruited in smaller numbers and to a more restricted area around the lesion; they were barely seen distal to the injury site. The decrease in T cell accumulation observed in the CNS from day 7 to day 21 after injury suggests that T cells may be eliminated by regulatory mechanisms. The nature of these accumulated T cells is unknown. However, it was shown in an experimental model of spinal cord injury that T cells isolated from spine-injured rats are capable of causing neurological deficits and histopathological changes similar to EAE when injected intravenously into naive animals. Disease induction was possible only when the T cells were obtained from rats 1 wk postinjury, suggesting that the encephalitogenic T cell repertoire triggered by the injury is under strict regulation (34). Thus, the accumulation of endogenous T cells after CNS axonal injury might be a reflection of a systemic immune response against self components exposed by the injury.

Our observation that death of infiltrating T cells occurs in the CNS after trauma and during spontaneous clinical recovery from EAE, but not in the PNS after trauma, supports the notion of immune privilege in the CNS (1, 12). The similarity in the extent of T cell death detected in injured rat optic nerves and in uninjured optic nerves of T_{MBP} -injected rats suggests that the mechanism of T cell elimination in the CNS is constitutive and is not dependent on injury. Thus, elimination of T cells through cell death appears to play a role in terminating immune reactions in the CNS, but not in the PNS. Similarly, T cell apoptosis was observed in EAE lesions (35–37). In cases of autoimmune disease, the CNS was indeed shown to have a high potential for elimination of T cells through a mechanism of apoptosis that is less

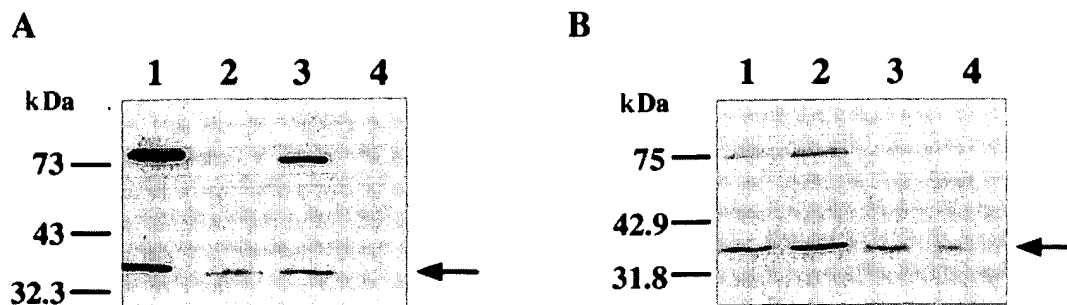


Figure 9. Western blot analysis of FasL expression in primary cultures of CNS glial cells and in optic and sciatic nerve high-speed supernatants. *A*) Qualitative analysis of glial cell lysates. Glial cells were cultured, differentiated *in vitro*, and harvested. Cell lysates of 1) astrocytes, 2) microglia, and 3) oligodendrocytes were prepared and subjected to Western blot analysis using monoclonal anti-FasL antibody, and 4) each sample was subjected to Western blot analysis using secondary antibody only. *B*) Quantitative analysis of injured and uninjured optic and sciatic nerve high-speed supernatants. High-speed supernatants of homogenized injured and uninjured optic and sciatic nerves were prepared and equal amounts of total protein from each sample of 1) uninjured optic nerve, 2) injured optic nerve, 3) uninjured sciatic nerve, and 4) injured sciatic nerve were subjected to Western blot analysis using monoclonal anti-FasL antibody. The arrow points to FasL protein in the cell membrane. Protein analysis of all samples with secondary antibody only was negative.

effective in the PNS and almost absent in other tissues such as muscle and skin (38). The present findings do not, however, exclude the possible operation of additional mechanisms of T cell regulation in the CNS after injury and in autoimmune disease. Tolerance of T cells in the CNS may be mediated by anergy or suppression in addition to elimination—for example, by a shift in the reactive T cell population from CD4+ Th1 cells [secreting interferon γ or interleukin 2 (IL-2)] to CD4+ Th2 cells (secreting IL-4 or IL-10), which are capable of suppressing the Th1 inflammatory response. Alternatively, T cell regulation could be controlled by antigen-presenting cells that do not possess the full cohort of secondary signals necessary to activate T cells (e.g., B7 costimulatory molecules). For example, MHC class II+/B7-microglia may ligate the T cell receptor without inducing T cell proliferation. This would result in functional inactivation of the T cells, or anergy (39).

The expression of MHC molecules is an important factor in the process of antigen recognition by T cells. MHC class II molecules are required for antigen presentation to helper T cells. In line with other studies (40, 41), we observed that MHC class II antigens (Ia) are constitutively expressed in the intact PNS but not in the intact CNS. In CNS white matter, MHC class II molecules appear to be inducible rather than constitutively expressed, i.e., their expression seems to be associated with injury. In the intact peripheral nerve, resident macrophages and fibroblasts are the best candidates to express MHC class II antigens (40, 41). Crushing of the peripheral nerve may also induce these antigens on Schwann cells (42), the main glial element in the PNS. In the CNS, expression of MHC molecules is undetectable immunohistochemically on both oligodendrocytes and neurons (7). Astrocytes and microglia might be the cells expressing MHC class II antigens after central nerve crush injury, as they are induced to

express Ia antigens and to function as antigen-presenting cells upon treatment with IFN- γ (9, 43–45). Nevertheless, even after injury, the expression of MHC class II antigens in the CNS is much weaker than in the PNS. This observation further highlights the distinct difference in immunological features between the PNS and the immune-privileged CNS.

The finding that optic nerve expresses FasL protein is in line with reported characteristics of immune-privileged sites and suggests that the Fas-FasL pathway may be involved in inducing death of infiltrating lymphocytes in the CNS, as described in the eye (28) and the testis (29). However, FasL is also expressed, albeit more weakly, in the sciatic nerve, indicating that FasL expression is not unique to immune-privileged sites. Moreover, we were unable to find direct evidence for Fas-mediated T cell cytotoxicity using glial cells expressing FasL. Some cells have indeed been shown to possess very high levels of surface FasL without being cytotoxic (46). Thus, expression of surface FasL may be a necessary but not a sufficient condition for Fas-mediated lysis. In addition, ligation of Fas on freshly isolated T cells has been shown to costimulate cellular activation and proliferation. It thus appears that Fas can mediate opposite effects, depending on the state of T cell activation (47). The differences in intensity and distribution of FasL expression between the injured and the uninjured optic nerve might be attributable to a differential subcellular localization of FasL. This would be in line with a recent report demonstrating that transport of Fas from cytoplasmic stores to the cell surface is an important mechanism in p53-mediated apoptosis (48). Therefore, it is possible that CNS injury and/or autoimmune inflammation can regulate sensitivity to apoptosis by allowing cytoplasmic death receptors to relocate to the cell surface. The observation of FasL expression in the *in vitro* primary cultures of CNS glial cells is consistent

with recent studies showing that FasL is constitutively present in human glial cells *in vivo* (49) and may contribute to the pathogenesis of multiple sclerosis (50).

Although many studies have pointed to a role for FasL in the control of immune responses by induction of apoptosis in infiltrating lymphocytes and granulocytes in the eye (28), testis (29), various murine and human tumors (51–53), and on thyrocytes in patients with Hashimoto's thyroiditis (54), some recent studies have questioned the immunoprotective effect of FasL. Allison et al. (55) reported that expression of FasL in the pancreatic islets of transgenic mice failed to protect these islets against allogeneic transplant rejection when placed under the kidneys of recipient mice. The same study demonstrated a proinflammatory function of FasL by induction of a potent granulocytic inflammatory response. Moreover, other recent studies (56, 57) have shown that mice deficient in Fas or FasL are resistant to induction of EAE, and that this is correlated with fewer inflammatory infiltrates and fewer cells undergoing apoptosis in the CNS of the mutant mice. It thus appears that FasL under certain circumstances can mediate apoptosis and under other circumstances can mediate activation and proliferation of immune cells. Taken together, these findings suggest that in the CNS FasL, possibly in conjunction with necessary partner molecules, might help to prevent immune responses by inducing the death of lymphocytes. Other possible functions of FasL, such as regulation of homeostasis or of stress responses, are not excluded. Additional studies are required to evaluate the specific role of FasL in the nervous tissue.

In conclusion, this study demonstrates that the immune-privileged CNS, in contrast to the PNS, uses both passive mechanisms (e.g., reduced expression of MHC class II antigens) and active mechanisms (e.g., death of infiltrating T cells) to limit the T cell immune response after injury and during spontaneous recovery from EAE. This limitation in T cell response may have apparently contradictory effects on the CNS. On the one hand, it prevents the development of massive inflammation and autoimmune diseases in the CNS. On the other hand, however, we have recently showed that increasing the autoimmune T cell response at a site of CNS injury can reduce the secondary degeneration of neurons after a primary axonal injury (58). Thus, immune privilege may be beneficial in protecting the CNS against remodeling of its neuronal network by limiting inflammation, but may be disadvantageous after injury when some immune responses are desirable for CNS recovery. **[F]**

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REFERENCES

1. Streilein, J. W. (1995) Unraveling immune privilege. *Science* **270**, 1158–1159
2. Ksander, B. R., and Streilein, J. W. (1994) Regulation of the immune response within privileged sites. *Chem. Immunol.* **58**, 117–145
3. Rao, K., Lund, R. D., Kunz, H. W., and Gill, T. J. D. (1989) The role of MHC and non-MHC antigens in the rejection of intracerebral allogeneic neural grafts. *Transplantation* **48**, 1018–1021
4. Poltorak, M., and Freed, W. J. (1991) BN rats do not reject F344 brain allografts even after systemic sensitization. *Ann. Neurol.* **29**, 377–388
5. Reese, T. S., and Karnovsky, M. J. (1967) Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J. Cell Biol.* **34**, 207–217
6. Barker, C. F., and Billingham, R. E. (1977) Immunologically privileged sites. *Adv. Immunol.* **25**, 1–54
7. Matsumoto, Y., Hara, N., Tanaka, R., and Fujiwara, M. (1986) Immunohistochemical analysis of the rat central nervous system during experimental allergic encephalomyelitis, with special reference to Ia-positive cells with dendritic morphology. *J. Immunol.* **136**, 3668–3676
8. Lassmann, H., Zimprich, F., Vass, K., and Hickey, W. F. (1991) Microglial cells are a component of the perivascular glia limitans. *J. Neurosci. Res.* **28**, 236–243
9. Wucherpfennig, K. W. (1994) Autoimmunity in the central nervous system: mechanisms of antigen presentation and recognition. *Clin. Immunol. Immunopathol.* **72**, 293–306
10. Cserr, H. F., and Knopf, P. M. (1992) Cervical lymphatics, the blood-brain barrier and the immunoreactivity of the brain: a new view. *Immunol. Today* **13**, 507–512
11. Wilbanks, G. A., and Streilein, J. W. (1992) Fluids from immune privileged sites endow macrophages with the capacity to induce antigen-specific immune deviation via a mechanism involving transforming growth factor-beta. *Eur. J. Immunol.* **22**, 1031–1036
12. Streilein, J. W. (1993) Immune privilege as the result of local tissue barriers and immunosuppressive microenvironments. *Curr. Opin. Immunol.* **5**, 428–432
13. Medawar, P. B. (1948) Immunity to homologous grafted skin III. The fate of skin homografts transplanted to the brain, to subcutaneous tissue and to the anterior chamber of the eye. *Br. J. Exp. Pathol.* **29**, 58–69
14. Cserr, H. F., Harling Berg, C. J., and Knopf, P. M. (1992) Drainage of brain extracellular fluid into blood and deep cervical lymph and its immunological significance. *Brain Pathol.* **2**, 269–276
15. Hickey, W. F., Hsu, B. L., and Kimura, H. (1991) T-lymphocyte entry into the central nervous system. *J. Neurosci. Res.* **28**, 254–260
16. Naporstek, Y., Ben Nun, A., Holoshitz, J., Reshef, T., Frenkel, A., Rosenberg, M., and Cohen, I. R. (1983) T lymphocyte lines producing or vaccinating against autoimmune encephalomyelitis (EAE). Functional activation induces peanut agglutinin receptors and accumulation in the brain and thymus of line cells. *Eur. J. Immunol.* **13**, 418–423
17. Perry, V. H., Brown, M. C., and Gordon, S. (1987) The macrophage response to central and peripheral nerve injury. A possible role for macrophages in regeneration. *J. Exp. Med.* **165**, 1218–1223
18. Hirschberg, D. L., Moalem, G., He, J., Mor, F., Cohen, I. R., and Schwartz, M. (1998) Accumulation of passively transferred

- primed T cells independently of their antigen specificity following central nervous system trauma. *J. Neuroimmunol.* **89**, 88–96
19. Hirshfeld, H., Teitelbaum, D., Arnon, R., and Sela, M. (1970) Basic encephalitogenic protein: a simplified purification on sulfoethyl-Sephadex. *FEBS Lett.* **7**, 317
20. Ben Nun, A., and Cohen, I. R. (1982) Experimental autoimmune encephalomyelitis (EAE) mediated by T cell lines: process of selection of lines and characterization of the cells. *J. Immunol.* **129**, 303–308
21. McCarthy, K. D., and de Vellis, J. (1980) Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell Biol.* **85**, 890–902
22. Raff, M. C., Miller, R. H., and Noble, M. (1983) A glial progenitor cell that develops *in vitro* into an astrocyte or an oligodendrocyte depending on culture medium. *Nature (London)* **303**, 390–396
23. Bottenstein, J. E., and Sato, G. H. (1979) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA* **76**, 514–517
24. Duvdevani, R., Rosner, M., Belkin, M., Sautter, J., Sabel, B. A., and Schwartz, M. (1990) Graded crush of the rat optic nerve as a brain injury model: combining electrophysiological and behavioral outcome. *Restor. Neurol. Neurosci.* **2**, 31–38
25. Yoles, E., Zalish, M., Lavie, V., Duvdevani, R., Ben-Bassat, S., and Schwartz, M. (1992) GM1 reduces injury-induced metabolic deficits and degeneration in the rat optic nerve. *Invest. Ophthalmol. Vis. Sci.* **33**, 3586–3591
26. Hirschberg, D. L., Yoles, E., Belkin, M., and Schwartz, M. (1994) Inflammation after axonal injury has conflicting consequences for recovery of function: rescue of spared axons is impaired but regeneration is supported. *J. Neuroimmunol.* **50**, 9–16
27. Hunig, T., Wallny, H.-J., Hartley, J. K., Lawetzky, A., and Tiefenthaler, G. (1989) A monoclonal antibody to a constant determinant of the rat T cell antigen receptor that induces T cell activation. *J. Exp. Med.* **169**, 73–86
28. Griffith, T. S., Brunner, T., Fletcher, S. M., Green, D. R., and Ferguson, T. A. (1995) Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* **270**, 1189–1192
29. Bellgrau, D., Gold, D., Selawry, H., Moore, J., Franzusoff, A., and Duke, R. C. (1995) A role for CD95 ligand in preventing graft rejection. *Nature (London)* **377**, 630–632
30. Tanaka, M., Suda, T., Takahashi, T., and Nagata, S. (1995) Expression of the functional soluble form of human fas ligand in activated lymphocytes. *EMBO J.* **14**, 1129–1135
31. Perry, V., Hume, D., and Gordon, S. (1985) Immunohistochemical localization of macrophages and microglia in the adult and developing brain. *Neuroscience* **15**, 313–326
32. Lazarov-Spiegler, O., Solomon, A. S., Zeev Brann, A. B., Hirschberg, D. L., Lavie, V., and Schwartz, M. (1996) Transplantation of activated macrophages overcomes central nervous system regrowth failure. *FASEB J.* **10**, 1296–1302
33. Hirschberg, D. L., and Schwartz, M. (1995) Macrophage recruitment to acutely injured central nervous system is inhibited by a resident factor: a basis for an immune-brain barrier. *J. Neuroimmunol.* **61**, 89–96
34. Popovich, P. G., Stokes, B. T., and Whitacre, C. C. (1996) Concept of autoimmunity following spinal cord injury: possible roles for T lymphocytes in the traumatized central nervous system. *J. Neurosci. Res.* **45**, 349–363
35. Pender, M. P., Nguyen, K. B., McCombe, P. A., and Kerr, J. F. (1991) Apoptosis in the nervous system in experimental allergic encephalomyelitis. *J. Neurol. Sci.* **104**, 81–87
36. Schmied, M., Breitschopf, H., Gold, R., Zischler, H., Rothe, G., Wekerle, H., and Lassmann, H. (1993) Apoptosis of T lymphocytes in experimental autoimmune encephalomyelitis. Evidence for programmed cell death as a mechanism to control inflammation in the brain. *Am. J. Pathol.* **143**, 446–452
37. Smith, T., Schmied, M., Hewson, A. K., Lassmann, H., and Cuzner, M. L. (1996) Apoptosis of T cells and macrophages in the central nervous system of intact and adrenalectomized Lewis rats during experimental allergic encephalomyelitis. *J. Autoimmun.* **9**, 167–174
38. Gold, R., Hartung, H. P., and Lassmann, H. (1997) T-cell apoptosis in autoimmune diseases: termination of inflammation in the nervous system and other sites with specialized immune-defense mechanisms. *Trends Neurosci.* **20**, 399–404
39. Schwartz, R. H. (1989) Acquisition of immunologic self-tolerance. *Cell* **57**, 1073–1081
40. Yu, L. T., Rostami, A., Silvers, W. K., Larossa, D., and Hickey, W. F. (1990) Expression of major histocompatibility complex antigens on inflammatory peripheral nerve lesions. *J. Neuroimmunol.* **30**, 121–128
41. Monaco, S., Gehrmann, J., Raivich, G., and Kreutzberg, G. W. (1992) MHC-positive, ramified macrophages in the normal and injured rat peripheral nervous system. *J. Neurocytol.* **21**, 623–634
42. Bergsteinsdottir, K., Kingston, A., and Jessen, K. R. (1992) Rat Schwann cells can be induced to express major histocompatibility complex class II molecules *in vivo*. *J. Neurocytol.* **21**, 382–390
43. Frei, K., Siepl, C., Groscurth, P., Bodmer, S., Schwerdel, C., and Fontana, A. (1987) Antigen presentation and tumor cytotoxicity by interferon-gamma-treated microglial cells. *Eur. J. Immunol.* **17**, 1271–1278
44. Fierz, W., Endler, B., Reske, K., Wekerle, H., and Fontana, A. (1985) Astrocytes as antigen-presenting cells. I. Induction of Ia antigen expression on astrocytes by T cells via immune interferon and its effect on antigen presentation. *J. Immunol.* **134**, 3785–3793
45. Fontana, A., Fierz, W., and Wekerle, H. (1984) Astrocytes present myelin basic protein to encephalitogenic T-cell lines. *Nature (London)* **307**, 273–276
46. Glass, A., Walsh, C. M., Lynch, D. H., and Clark, W. R. (1996) Regulation of the Fas lytic pathway in cloned CTL. *J. Immunol.* **156**, 3638–3644
47. Lynch, D. H., Ramsdell, F., and Alderson, M. R. (1995) Fas and FasL in the homeostatic regulation of immune responses. *Immunol. Today* **16** (12), 569–574
48. Bennett, M., Macdonald, K., Chan, S.-W., Luzio, J. P., Simari, R., and Weissberg, P. (1998) Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science* **282**, 290–293
49. Bonetti, B., and Raine, C. S. (1997) Multiple sclerosis: oligodendrocytes display cell death-related molecules *in situ* but do not undergo apoptosis. *Ann. Neurol.* **42**, 74–84
50. D'Souza, S. D., Bonetti, B., Balasingam, V., Cashman, N. R., Barker, P. A., Trout, A. B., Raine, C. S., and Antel, J. P. (1996) Multiple sclerosis: Fas signaling in oligodendrocyte cell death. *J. Exp. Med.* **184**, 2361–2370
51. Hahne, M., Rimoldi, D., Schroter, M., Romero, P., Schreier, M., French, L. E., Schneider, P., Bornand, T., Fontana, A., Lienard, D., Cerottini, J., and Tschopp, J. (1996) Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. *Science* **274**, 1363–1366
52. Niehans, G. A., Brunner, T., Frizelle, S. P., Liston, J. C., Salerno, C. T., Knapp, D. J., Green, D. R., and Kratzke, R. A. (1997) Human lung carcinomas express Fas ligand. *Cancer Res.* **57**, 1007–1012
53. O'Connell, J., O'Sullivan, G. C., Collins, J. K., and Shanahan, F. (1996) The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. *J. Exp. Med.* **184**, 1075–1082
54. Giordano, C., Stassi, G., De Maria, R., Todaro, M., Richiusa, P., Papoff, G., Ruberti, G., Bagnasco, M., Testi, R., and Galluzzo, A. (1997) Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis. *Science* **275**, 960–963
55. Allison, J., Georgiou, H. M., Strasser, A., and Vaux, D. L. (1997) Transgenic expression of CD95 ligand on islet beta cells induces a granulocytic infiltration but does not confer immune privilege upon islet allografts. *Proc. Natl. Acad. Sci. USA* **94**, 3943–3947
56. Sabelko, K. A., Kelly, K. A., Nahm, M. H., Cross, A. H., and Russell, J. H. (1997) Fas and Fas ligand enhance the pathogenesis of experimental allergic encephalomyelitis, but are not essential for immune privilege in the central nervous system. *J. Immunol.* **159**, 3096–3099
57. Waldner, H., Sobel, R. A., Howard, E., and Kuchroo, V. K. (1997) Fas- and FasL-deficient mice are resistant to induction of autoimmune encephalomyelitis. *J. Immunol.* **159**, 3100–3103
58. Moalem, G., Leibowitz-Amit, R., Yoles, E., Mor, F., Cohen, I. A., and Schwartz, M. (1999) Autoimmune T cells protect neurons from secondary degeneration following central nervous system axotomy. *Nature Med.* **5**, 49–55

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The neuroprotective effect of inflammation: implications for the therapy of multiple sclerosis

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Abstract

Autoreactive T cells are a component of the normal immune system. It has been proposed that some of these autoreactive T cells even have a protective function. Recent studies support this notion by demonstrating that (a) myelin basic-protein (MBP-) specific T cells show neuroprotective effects *in vivo*, and (b) activated antigen-specific human T cells and other immune cells produce bioactive brain-derived neurotrophic factor (BDNF) *in vitro*. Furthermore, BDNF is expressed in different types of inflammatory cells in brain lesions of patients with acute disseminated leukoencephalopathy or multiple sclerosis. We postulate that the neuroprotective effect of T cells and other immune cells observed *in vivo* is at least partially mediated by BDNF and other neurotrophic factors. The concept of neuroprotective autoimmunity has obvious implications for the therapy of multiple sclerosis and other neuroimmunological diseases. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Autoimmunity; Immunotherapy; Multiple sclerosis; Neuroprotection; Neurotrophic factors

1. Introduction

It has long been known that in health and disease, the immune system and the nervous system are closely linked at different levels. For example, when immune cells attack the nervous system, neuroimmunological diseases arise. Multiple sclerosis and its animal models provide the paradigm for such a deleterious interaction between cells of the immune and nervous system. Experimental autoimmune encephalomyelitis (EAE) can be induced by active immunization with CNS autoantigens [e.g., myelin basic protein (MBP)], or by the transfer of autoantigen-specific T cells into naïve syngeneic recipients (Wekerle et al., 1994). Recently it was demonstrated that MBP-specific T cells may have — seemingly paradoxical — neuroprotective (side-)effects (Moalem et al., 1999; Schwartz et al., 1999). The mechanisms of the neuroprotective effects of T cells and other immune cells are presently unknown.

A different line of research has revealed that unexpect-

edly, T cells and other cells of the immune system are capable of producing neurotrophic factors (Torcia et al., 1996; Besser and Wank, 1999; Kerschensteiner et al., 1999). Here we propose that the two lines of investigation converge: The observed neuroprotective effects of immune cells may at least partially be mediated by their production and local secretion of neurotrophic factors. This concept would have far reaching consequences for the therapy of neuroimmunological diseases, especially multiple sclerosis.

2. Evidence for a neuroprotective effect of T cells and other immune cells

Autoimmune T cells have been shown to protect neurons from secondary degeneration after a partial crush injury of the optic nerve (Moalem et al., 1999). In a series of elegant experiments, T cells specific for MBP, ovalbumin (OVA), or a heatshock protein (hsp) peptide were activated with their respective antigens *in vitro*, and then injected intraperitoneally into rats immediately after unilateral optic nerve injury. Seven days after injury, the optic nerves were analysed immunohistochemically for the

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presence of T cells. Small numbers of T cells were observed in the intact (uninjured) optic nerves of rats injected with anti-MBP T cells (this would be consistent with previous observations that anti-MBP T cells home to intact CNS white matter (Naparstek et al., 1983)). A much more pronounced accumulation of T cells, however, was observed in the *crushed optic nerves* of the rats injected with T cells specific for MBP, hsp peptide, or OVA.

The degree of primary and secondary damage to the optic nerve axons and their attached retinal ganglion cells was measured by injecting a neurotracer distal to the site of the optic nerve lesion immediately after the injury, and again after two weeks (Moalem et al., 1999). The percentage of labeled retinal ganglion cells (reflecting viable axons) was significantly greater in the retinas of the rats injected with anti-MBP T cells than in the retinas of rats injected with anti-OVA or anti-hsp peptide T cells. Thus, although all three T-cell lines accumulated at the site of injury, only the MBP-specific, autoimmune T cells had a substantial effect in limiting the extent of secondary degeneration.

The neuroprotective effect was confirmed by electrophysiological studies. On day 14 after injury, the mean compound action potential amplitude of the distal segment recorded from the injured nerves obtained from rats injected with the anti-MBP T cells was 2.5 times larger than the potential recorded in control rats. The results demonstrate that T-cell autoimmunity can mediate significant neuroprotection after CNS injury. The authors speculate that after injury, 'cryptic' epitopes might become available and might be recognized by endogenous nonencephalitogenic (benign) T cells. After local stimulation, these protective autoreactive T cells could exert their neuroprotective effect. The findings further substantiate the idea that 'natural autoimmunity' can be benign and may even function as a protective mechanism (Cohen, 1992).

Macrophages seem to represent another type of immune cell that is capable of mediating neuroprotection and/or stimulating recovery: The injection of activated macrophages into transected rat spinal cord stimulated tissue repair and partial recovery of motor function (Rapalino et al., 1998). The neuroprotective activity of immune cells is not restricted to the CNS. After experimental axotomy of the facial nerve of immunodeficient SCID mice, the survival of facial motor neurons was severely impaired compared to immunocompetent wild type mice (Serpe et al., 1999). Reconstitution of SCID mice with wild-type splenocytes containing T and B cells restored the survival of facial motor neurons in these mice to the level of the wild-type controls.

3. Evidence for the production of neurotrophic factors by immune cells

Outside the nervous system, several types of immune cells and hematogenic precursor cells have been shown to

express one or more neurotrophic factors. For example, nerve growth factor (NGF) is produced by B cells, which also express the trkA receptor and p75 NGF receptor (Torcia et al., 1996). Because neutralization of endogenous NGF caused apoptosis of memory B cells, it was concluded that NGF is an autocrine growth factor for memory B cells (Torcia et al., 1996). NGF has been detected also in T cells, although a functional role could not be demonstrated (Ehrhard et al., 1993; Santambrogio et al., 1994).

More recently, another neurotrophin, brain-derived neurotrophic factor (BDNF), was found to be expressed in immune cells. BDNF was originally cloned in 1989 (Leibrock et al., 1989) as the second member of the neurotrophin family which includes nerve growth factor (NGF) and neurotrophins (NT)-3, -4/5, -6 and -7 (Lewin and Barde, 1996). Since then, the important role of BDNF in regulating the survival and differentiation of various neuronal populations including sensory neurons, cerebellar neurons and spinal motor neurons has been firmly established (Lewin and Barde, 1996). Neurons are the major source of BDNF in the nervous system (Hofer et al., 1990; Lewin and Barde, 1996). It is thought that BDNF and NT4/5 exert their biological function via the full-length form of trkB receptor called gp145trkB (Klein et al., 1991), expression of which seems to be restricted to neuronal cell populations (Lomen-Hoerth and Shooter, 1995).

In a recent paper, we demonstrated that activated human T cells, B cells and monocytes secrete bioactive BDNF *in vitro* (Kerschensteiner et al., 1999). Notably, in Th1- and Th2-type CD4⁺ T cell lines specific for myelin autoantigens such as MBP or myelin-oligodendrocyte glycoprotein (MOG), BDNF production is increased upon antigen stimulation. The BDNF secreted by immune cells is bioactive as it supports neuronal survival *in vitro* (Kerschensteiner et al., 1999). Thus far, however, we found no evidence that BDNF acts on peripheral human immune cells. This could be explained by the fact that immune cells mostly express the truncated form of the gp95trkB BDNF receptor [previous reports indicated that the expression of the full-length, signal-transducing form of gp145trkB is largely restricted to neuronal cells, whereas the truncated gp95trkB form is widely expressed in non-neuronal tissues (Lomen-Hoerth and Shooter, 1995)]. Clearly, the question whether BDNF can affect elements of the immune system deserves more detailed study.

Similar observations were recently reported by several other groups of investigators. After experimental injury of the striatum activated macrophages and microglia cells transcribe mRNA for glial cell line-derived neurotrophic factor (GDNF) and BDNF (Batchelor et al., 1999). This could help to explain the sprouting of dopaminergic neurons observed after experimental injury (Batchelor et al., 1999). Further, BDNF was detected in activated microglia in the brain lesions of patients with human

immunodeficiency virus (HIV) type 1-associated encephalitis (Soontornniyomkij et al., 1999).

Transcripts for BDNF and neurotrophin-3 and their receptors *trkB* and *trkC* were found in subpopulations of human peripheral blood cells (Besser and Wank, 1999). BDNF protein was secreted by cultured T cell clones. However, a functional effect was not demonstrated, although the authors detected transcripts for the full-length *trkB* receptor in some, especially Th1 type T-cell clones (Besser and Wank, 1999). Again, it would be premature to dismiss the possibility that neurotrophins have a functional role in the immune system. They might be expressed not only in circulating immune cells but also in resident cells, including stromal elements of lymphoid organs. Consistent with this notion, several neurotrophins and their receptors have recently been demonstrated in human bone marrow (Labouyrie et al., 1999). More types of immune cells need to be examined for possible functional effects of neurotrophins.

4. Expression of BDNF in multiple sclerosis lesions

We hypothesize that the neurotrophic effects of immune cells demonstrated *in vivo* (Moalem et al., 1999; Schwartz et al., 1999) are at least partially mediated by neurotrophic factors 'imported' into the CNS by activated immune cells (Besser and Wank, 1999; Kerschensteiner et al., 1999). Are the postulated neuroprotective effects of inflammation

relevant to human disease? As a first step to answer this question, we searched for the expression of neurotrophins in inflammatory brain lesions of patients with multiple sclerosis and postinfectious acute disseminated leukoencephalitis. Using four different monoclonal antibodies and a polyclonal anti-BDNF antiserum, we could indeed demonstrate that BDNF is expressed in different types of immune cells at different sites in inflammatory lesions (Kerschensteiner et al., 1999). BDNF immunoreactivity was observed in inflammatory cells forming perivascular infiltrates in cases of disseminated (postinfectious) leukoencephalitis and multiple sclerosis (Fig. 1). Using serial sections, BDNF-positive cells were found to correspond to infiltrating mononuclear cells. In multiple sclerosis, BDNF-positive lymphocytes and macrophages were not restricted to the perivascular localization but found throughout the lesion (Fig. 1). Lesional areas with high numbers of macrophages actively involved in demyelination showed enhanced BDNF immunoreactivity. In inflammatory CNS disease and healthy controls, BDNF was also detected in various types of neurons, ependymal cells, and weakly in astrocytes. No immunoreactivity was observed in oligodendrocytes or ramified microglial cells.

What are the possible consequences of neurotrophic factors expressed by immune cells in inflammatory lesions? There are several possibilities. First, the 'imported' BDNF could support neuronal survival and enhance neuronal and axonal repair. If this mechanism occurs in multiple sclerosis lesions, it is clearly insufficient in the long run, as axonal and neuronal injury are important features of the

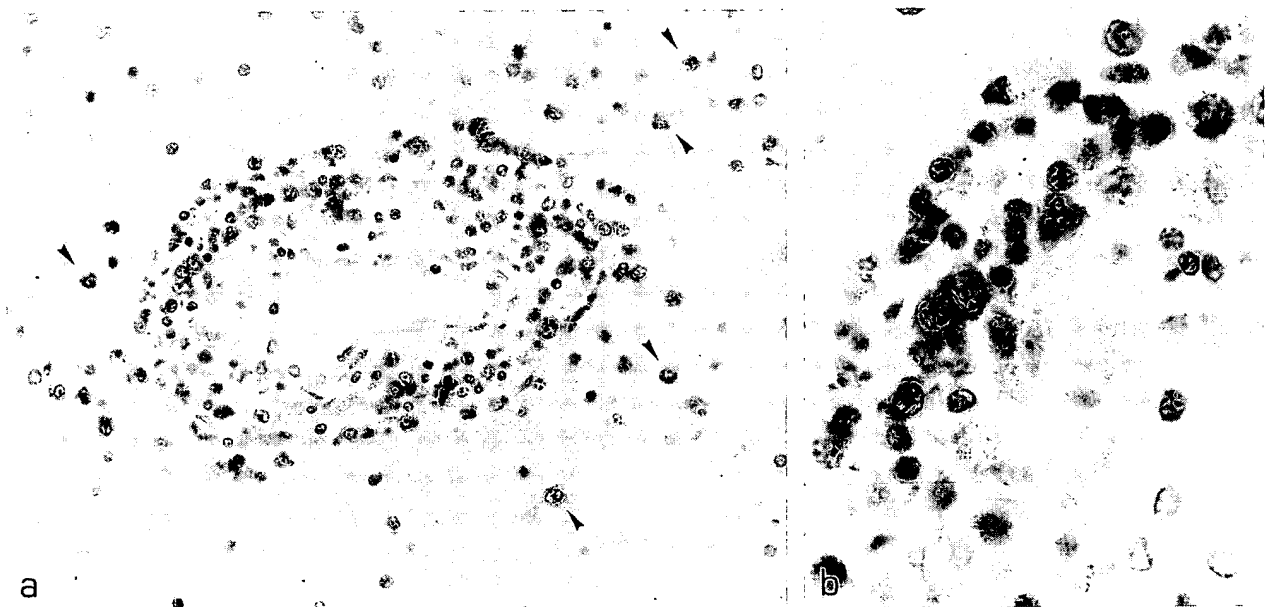


Fig. 1. Immunolocalization of BDNF in inflammatory brain lesions in multiple sclerosis. a,b: Perivascular infiltrate in a multiple sclerosis plaque containing inflammatory cells stained with a monoclonal antibody against BDNF (red). BDNF-positive mononuclear cells are found not only at perivascular sites, but also in the plaque parenchyma (arrows); (a: $\times 400$, b, $\times 1000$).

pathology (Ferguson et al., 1997; Trapp et al., 1998). Second, locally produced neurotrophins might affect remyelination. Indeed, BDNF and NT-3 have been shown to induce oligodendrocyte proliferation and myelination of regenerating axons in experimental spinal cord lesions (McTigue et al., 1998). Third, the neurotrophins produced by inflammatory cells could have beneficial immunoregulatory effects, either by acting on other inflammatory cells or on microglia and astroglia. Several neurotrophic factors, including BDNF, were shown to inhibit the inducibility of MHC class II on microglia (Neumann et al., 1998). Further, some neurotrophic factors downregulate costimulatory molecules on microglia (Wei and Jonakait, 1999).

The concept of the neuroprotective role of inflammation can be extended to neurodegenerative, ischemic and traumatic lesions of the nervous system, considering that inflammation is a universal tissue reaction crucial for defense and repair (Wekerle et al., 1986; Perry et al., 1993; Moser, 1997; Hirschberg et al., 1998; Ransohoff and Tani, 1998).

5. Implications for therapy

According to current opinion, the treatment of multiple sclerosis has two major objectives, namely (a) suppression of the inflammatory process, and (b) restoration and protection of glial and neuronal function (Compston, 1994). The potential neuroprotective function of inflammatory cells is relevant to both these treatment goals.

5.1. 'Import' of neurotrophic factors via immune cells

A number of studies have shown that the administration of BDNF protein or the BDNF gene can rescue injured or degenerating neurons and induce axonal outgrowth and regeneration (Yan et al., 1992; Thoenen et al., 1994; Gravel et al., 1997; Kobayashi et al., 1997; McTigue et al., 1998). Furthermore, BDNF had beneficial effects in several animal models of neurodegenerative diseases (Mitsumoto et al., 1994). Difficulties in delivering sufficient amounts of BDNF to the site of CNS lesions have so far hampered the successful application of BDNF and other neurotrophic factors for treatment of human diseases (Sagot et al., 1997). One promising novel strategy for the delivery of neuroprotective factors relies on the (retroviral) transduction of one or several neurotrophic factors into antigen-specific T cell lines (Kramer et al., 1995). As the transduced T cells are specific for an autoantigen expressed in the nervous system, they home to the sites where the relevant autoantigen is expressed, recognize their antigen and are then stimulated locally to secrete neurotrophic factor(s) (Kramer et al., 1995). The results discussed here indicate that this experimental strategy has a natural

counterpart. However, it appears that the neurotrophins secreted by immune cells under natural conditions are often insufficient to prevent injury. It will therefore be worthwhile to further refine the strategies to transduce neurotrophic factors into immune cells and to exploit the homing properties of the immune cells for targeting neuroprotective factors into the nervous system (Kramer et al., 1995; Flügel et al., 1999).

5.2. Implications for immunosuppressive and immunomodulatory therapy

It seems likely that the nonselective immunosuppressive treatments that have been used for the treatment of multiple sclerosis eliminate the neuroprotective (benign) autoimmune cells along with the autoaggressive offenders. This may be one of the reasons why treatment with nonselective immunosuppressive agents often fails to have a convincing clinical benefit (Hohlfeld, 1997; Noseworthy et al., 1999). Undebatably, there is a convincing rationale for immunosuppressive treatment in situations when the noxious effects of inflammation prevail. However, immunosuppressive therapy is likely to fail when the beneficial effects of the inflammatory reaction outweigh its negative consequences. In multiple sclerosis it is unfortunately unclear whether there is a stage of the disease when the inflammatory reaction is more beneficial than harmful. Further studies of the expression of BDNF and other neurotrophins and their receptors in multiple sclerosis lesions may help to define such stage(s).

More recent treatment strategies, most of them still experimental, attempt to modulate the autoimmune reaction selectively. The proposed treatments target various immune molecules, such as cytokines, adhesion molecules, co-stimulatory molecules and last but not least, the trimolecular complex of T-cell recognition (e.g., by application of altered peptide ligands or by vaccination with T cells or T-cell receptor peptides; reviewed in (Hohlfeld, 1997)). Autoreactive, myelin-specific T cells are known to be functionally heterogeneous (Meinl et al., 1997). In the future, it will be important to learn how to preserve or even enhance the proposed neuroprotective function of the 'benign' autoreactive T cells during immunomodulatory intervention.

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References

- Batchelor, P.E., Liberatore, G.T., Wong, J.Y.F., Porritt, M.J., Frerichs, F., Donnan, G.A. et al., 1999. Activated macrophages and microglia induce dopaminergic sprouting in the injured striatum and express brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. *J. Neurosci.* 19, 1708–1716.
- Besser, M., Wank, R., 1999. Clonally restricted production of the neurotrophins, brain-derived neurotrophic factor and neurotrophin-3 mRNA by human immune cells and Th1/Th2-polarized expression of their receptors. *J. Immunol.* 162, 6303–6306.
- Cohen, I.R., 1992. The cognitive paradigm and the immunological homunculus. *Immunol. Today* 13, 490–494.
- Compston, A., 1994. Future prospects for the management of multiple sclerosis. *Ann. Neurol.* 36, S146–S150.
- Ehrhard, P.B., Erb, P., Graumann, U., Otten, U., 1993. Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. *Proc. Natl. Acad. Sci. USA* 90, 10984–10988.
- Ferguson, B., Matyszak, M.K., Esiri, M.M., Perry, V.H., 1997. Axonal damage in acute multiple sclerosis lesions. *Brain* 120, 393–399.
- Flügel, A., Willem, M., Berkowicz, T., Wekerle, H., 1999. Gene transfer into CD4⁺ T lymphocytes: Green fluorescent protein engineered, encephalitogenic T cells used to illuminate immune responses in the brain. *Nature Med.* 5, 843–847.
- Gravel, C., Götz, R., Lorrain, A., Sendtner, M., 1997. Adenoviral gene transfer of ciliary neurotrophic factor and brain-derived neurotrophic factor leads to long-term survival of axotomized motor neurons. *Nature Med.* 3, 765–769.
- Hirschberg, D.L., Moalem, G., He, J., Mor, F., Cohen, I.R., Schwarz, M., 1998. Accumulation of passively transferred primed T cells independently of their antigen specificity following nervous system trauma. *J. Neuroimmunol.* 89, 88–96.
- Hofer, M., Pagliusi, S.R., Hohn, A., Leibrock, A., Barde, Y.-A., 1990. Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *EMBO J.* 9, 2459–2464.
- Hohlfeld, R., 1997. Biotechnological agents for the immunotherapy of multiple sclerosis. Principles, problems and perspectives. *Brain* 120, 865–916.
- Kerscheneitner, M., Gallmeier, E., Behrens, L., Klinkert, W.E.F., Kolbeck, R., Hoppe, E. et al., 1999. Activated human T cells, B cells and monocytes produce brain-derived neurotrophic factor (BDNF) in vitro and in brain lesions: A neuroprotective role of inflammation? *J. Exp. Med.* 189, 865–870.
- Klein, R., Nanduri, V., Jing, S., Lamballe, F., Tapley, P., Bryant, S. et al., 1991. The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell* 66, 395–403.
- Kobayashi, N.R., Fan, D.-P., Giehl, K.M., Bedard, A.M., Wiegand, S.J., Tetzlaff, W., 1997. BDNF and NT-4/5 prevent atrophy of rat rubrospinal neurons after cervical axotomy, stimulate GAP-43 and α 1-tubulin mRNA expression, and promote axonal regeneration. *J. Neurosci.* 17, 9583–9595.
- Kramer, R., Zhang, Y., Gehrman, J., Gold, R., Thoenen, H., Wekerle, H., 1995. Gene transfer through the blood–nerve barrier: Nerve growth factor engineered neuritic T lymphocytes attenuate experimental autoimmune neuritis. *Nature Med.* 1, 1162–1166.
- Labouyrie, E., Dubus, P., Groppi, A., Mahon, F.X., Ferrer, J., Parrens, M. et al., 1999. Expression of neurotrophins and their receptors in human bone marrow. *Am. J. Pathol.* 154, 405–415.
- Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P. et al., 1989. Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* 341, 149–152.
- Lewin, G.R., Barde, Y.-A., 1996. Physiology of the neurotrophins. *Annu. Rev. Neurosci.* 19, 289–317.
- Lomen-Hoerth, C., Shooter, E.M., 1995. Widespread neurotrophin receptor expression in the immune system and other nonneuronal rat tissues. *J. Neurochem.* 64, 1780–1789.
- McTigue, D.M., Horner, P.J., Stokes, B.T., Gage, F.H., 1998. Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. *J. Neurosci.* 18, 5354–5365.
- Meinl, E., Hoch, R.M., Dornmair, K., De Waal Malefijt, R., Bontrop, R.E. et al., 1997. Encephalitogenic potential of myelin basic protein-specific T cells isolated from normal rhesus macaques. *Am. J. Pathol.* 150, 445–453.
- Mitsumoto, H., Ikeda, K., Klinkosz, B., Cedarbaum, J.M., Wong, V., Lindsay, R.M., 1994. Arrest of motor neuron disease in wobbler mice cotreated with CNTF and BDNF. *Science* 265, 1107–1110.
- Moalem, G., Leibowitz-Amit, R., Yoles, E., Mor, F., Cohen, I.R., Schwartz, M., 1999. Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nature Med.* 5, 49–55.
- Moser, H.W., 1997. Adrenoleukodystrophy: Phenotype, genetics, pathogenesis and therapy. *Brain* 120, 1485–1508.
- Napartek, Y., Ben-Nun, A., Holoshitz, J., Reshef, T., Frenkel, A., Rosenberg, M. et al., 1983. T lymphocyte line producing or vaccinating against autoimmune encephalomyelitis (EAE). Functional activation induces peanut agglutinin receptors and accumulation in the brain and thymus of line cells. *Eur. J. Immunol.* 13, 418–423.
- Neumann, H., Misgeld, T., Matsumuro, K., Wekerle, H., 1998. Neurotrophins inhibit class II inducibility of microglia: Involvement of the p75 receptor. *Proc. Natl. Acad. Sci. USA* 95, 5779–5784.
- Noseworthy, J.H., Gold, R., Hartung, H.-P., 1999. Treatment of multiple sclerosis: Recent trials and future perspectives. *Curr. Opin. Neurol.* 12, 279–293.
- Perry, V.H., Andersson, P.-B., Gordon, S., 1993. Macrophages and inflammation in the central nervous system. *Trends Neurosci.* 16, 268–273.
- Ransohoff, R.M., Tani, M., 1998. Do chemokines mediate leukocyte recruitment in post-traumatic CNS inflammation? *Trends Neurosci.* 21, 154–159.
- Rapalino, O., Lazarov-Spiegler, O., Agranov, E., Velan, G.J., Yoles, E., Fraidakis, M. et al., 1998. Implantation of stimulated homologous macrophages results in partial recovery of paraplegic rats. *Nature Med.* 4, 814–821.
- Sagot, Y., Vejsada, R., Kato, A.C., 1997. Clinical and molecular aspects of motor-neurone diseases: Animal models, neurotrophic factors and Bcl-2 oncoprotein. *Trends Pharmacol. Sci.* 18, 330–337.
- Santambrogio, L., Benedetti, M., Chao, M.V., Muzaffar, R., Kulig, K., Gabelli, N. et al., 1994. Nerve growth factor production by lymphocytes. *J. Immunol.* 153, 4488–4495.
- Schwartz, M., Moalem, G., Leibowitz-Amit, R., Cohen, I.R., 1999. Innate and adaptive immune responses can be beneficial for CNS repair. *Trends Neurosci.* 22, 295–299.
- Serpe, C.J., Kohm, A.P., Huppenbauer, C.B., Sanders, V.J., Jones, K.J., 1999. Exacerbation of facial motor-neuron loss after facial nerve transection in severe combined immunodeficient (SCID) mice. *J. Neurosci.* 19, 1–5.
- Soontornniyomkij, V., Wang, G., Pittman, C.A., Wiley, C.A., Achim, C.L., 1999. Expression of brain-derived neurotrophic factor protein in activated microglia of human immunodeficiency virus type 1 encephalitis. *Neuropathol. Appl. Neurobiol.* 24, 453–460.
- Thoenen, H., Castrén, E., Berzaghi, M., Blöchl, A., Lindholm, D., 1994. In: Racagni, G., Brunello, N., Langer, S.Z. (Eds.), *Recent Advances in the Treatment of Neurodegenerative Disorders and Cognitive Dysfunction*, Karger, Basel, pp. 197–203.
- Torcia, M., Bracci-Laudiero, L., Lucibello, M., Nencioni, L., Labardi, D., Rubatelli, A. et al., 1996. Nerve growth factor is an autocrine survival factor for memory B lymphocytes. *Cell* 85, 345–356.
- Trapp, B.D., Peterson, J., Ransohoff, R.M., Rudick, R., Mörk, S., Bö, L., 1998. Axonal transection in the lesion of multiple sclerosis. *N. Engl. J. Med.* 338, 278–285.
- Wei, R.T., Jonakait, G.M., 1999. Neurotrophins and the anti-inflammatory agents interleukin-4 (IL-4), IL-10, IL-11 and transforming growth

- factor-beta 1 (TGF-beta 1) down-regulate T cell costimulatory molecules B7 and CD40 on cultured rat microglia. *J. Neuroimmunol.* 95, 8–18.
- Wekerle, H., Kojima, K., Lannes-Vieira, J., Lassmann, H., Linington, C., 1994. Animal models. *Ann. Neurol.* 36, S47–S53.
- Wekerle, H., Linington, C., Lassmann, H., Meyermann, R., 1986. Cellular immune reactivity within the CNS. *Trends Neurosci.* 9, 271–277.
- Yan, Q., Elliott, J., Snider, W.D., 1992. Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. *Nature* 360, 753–755.

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